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RESEARCH DIRECTED TOWARD DEVELOPMENT OF TEST PROCEDURES FOR EVALUATING ALLOWABLE LIMITS OF CONCENTRATION OF TOXIC SUBSTANCES IN AQUATIC ENVIRONMENTS

Engineering Science, Incorporated
Oakland, California

30 November 1962

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FINAL REPORT

PROJECT YEAR 1961-62

RESEARCH DIRECTED TOWARD DEVELOPMENT OF TEST PROCEDURES FOR EVALUATING ALLOWABLE LIMITS OF CONCENTRATION OF TOXIC SUBSTANCES IN AQUATIC ENVIRONMENTS

PREPARED FOR

DIVISION OF WATER SUPPLY AND POLLUTION CONTROL
U.S. PUBLIC HEALTH SERVICE

ENGINEERING-SCIENCE, INC.

30 NOVEMBER, 1962

HEAD OFFICE
150 E. FOOTHILL BLVD.
ARCADIA, CALIFORNIA

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4144 TELEGRAPH AVE.
OAKLAND, CALIFORNIA

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ENGINEERING-SCIENCE, INC.
ENGINEERS AND CONSULTANTS

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30 November 1962

Mr. Bernard B. Berger
Sanitary Engineer, Chief
Water Supply and Water Pollution Research
Robert A. Taft Sanitary Engineering Center
4676 Columbia Parkway
Cincinnati 26, Ohio

Dear Mr. Berger:

This will transmit our final report pursuant to Contract No. PH 86-62-55, dated 1 December 1961 for research on the development of means of detecting and quantifying the effects of sublethal concentrations of toxic substances on aquatic organisms. The efforts of this twelve month period have been devoted to the search for, and the development of, bioassay procedures having significantly greater sensitivity than those presently available.

The several most promising bioassay methods developed as a result of this comprehensive study were tested in a preliminary manner to confirm their biological validity, with progressive improvement in instrumentation concomitantly occurring. The measures are now ready for intensive examination in depth, and following this the quantitative evaluation of the toxic effects of selected water pollutants can begin.

Sincerely yours,

Harvey F. Ludwig

Harvey F. Ludwig
ENGINEERING-SCIENCE, INC.

FINAL REPORT

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Prepared For

Division of Water Supply and Pollution Control
U.S. Public Health Service

ENGINEERING-SCIENCE, INC.

30 November 1962

Head Office:
150 E. Foothill Blvd.
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I. INTRODUCTION

THE REPORT

This final report summarizes research on the bioassay aspects of aquatic toxicology conducted during the period 1 December 1961 - 30 November 1962 by Engineering-Science, Inc. This work was done under contract with the division of Water Supply and Pollution Control, U.S. Public Health Service. The report will present research findings and conclusions, together with descriptions of new apparatus to the extent that their descriptions are judged useful to researchers and others interested in this approach to the study of living organisms.

Because the individual tests contained elements both of independence and interdependence with each other, the research findings reported herein are presented within their individual experimental frameworks. A separate section on Conclusions and Recommendations summarizes and integrates the salient features of all experiments.

In general, the interpretations of data and conclusions should be considered as preliminary, their primary function that of guiding the more definitive research now underway. In most instances, the experiments reported herein were designed to test the amenability of a specific behavioral or physiological parameter of toxic response to quantitative bioassay procedures. In some cases the results were encouraging, in others not so. Only those aspects which clearly demonstrated features permitting quantification of biologically significant response mechanisms were carried beyond a cursory examination.

PROJECT STAFF

Engineering-Science, Inc. staff for the study included Mr. Harvey F. Ludwig as Project Director, and for the first quarter of the year Dr. Mervin J. Stewart as Project Supervisor. During the latter three quarters of the project year, Richard E. Warner assumed supervision of the study as Project Biologist. Miss Ethelle Rothman and Mr. Okpo Onykwere conducted parts of the laboratory experimentation. James Thorne contributed valuable theoretical and biophysical ideas, and worked closely with the Project Biologist on several experimental procedures. Ralf C. Carter provided significant assistance in instrumentation.

ACKNOWLEDGEMENTS

Many individuals contributed time and energy toward this project in the way of ideas, criticisms, suggestions, and guidance in maintaining the desired orientation of the project. Important assistance was received from Drs. Peter Doudoroff, Alfred Bartsch, Donald Mount, and H. Page Nicholson of the U.S. Public

Health Service with suggestions, criticisms, and availability of results of their previous researches. Dr. Erman Pearson, Department of Sanitary Engineering, University of California, provided guidance and helpful suggestions. Dr. Mark R. Rosenzweig, Department of Psychology, University of California, kindly reviewed experimental data and discussed theoretical aspects of conditioned responses with the staff; while Dr. Elizabeth Scott, Department of Statistics, University of California, generously provided help in developing statistical designs and analysing response data. Drs. Cadet Hand, Ralph I. Smith, Paul Needham and A. Starker Leopold, Department of Zoology, University of California, contributed significant ideas and were of assistance in maintaining the biological orientation of the study.

While these and other contributors--all of whom have the sincere gratitude of the staff--were essential to the progress made, the staff alone is responsible for the interpretations of data and any errors of omission or commission.

II. GENERAL CONSIDERATIONS

FRAME OF REFERENCE

With the steady growth of the biological sciences, the field of pollution biology has become progressively more diversified. Several alternate philosophies of approach are now available to workers dealing with the basic and applied aspects of the field. Actually, a researcher can do productive work in pollution biology within the framework of a wide variety of biological fields. Nevertheless, there seem to be developing two broad approaches to the problem. These two approaches will be reviewed briefly in order to place the Engineering-Science, Inc. research project in proper perspective. It will be seen that both of these general approaches have merits peculiar to themselves, as well as inherent limitations.

The Field Approach

The effects of pollution are assessed in the field, generally a posteriori. Changes in species composition, population abundance, and the general character of the ecosystem are determined, usually, by a number of established sampling techniques. Typical of this approach is the thorough paper of Cushing and Olive (1957) entitled Effects of toxaphene and rotenone upon the macroscopic bottom fauna of two northern Colorado reservoirs.

Included within the field approach, which might with equal ease be labelled the ecological approach, is the "biological index" concept. Here the relative abundance of selected species constellations are analyzed, generally over time. Patrick's studies of diatoms as indicators of changes in environmental conditions (e. g. 1957) demonstrates the general nature of the application of this concept.

It is important to recognize here that the researcher who works within an existing ecological framework deals with the interactions of multifarious populations, usually with time as one parameter. He is therefore largely independent of considerations of toxic effect at the individual level.

The field approach has the distinct merit of dealing with pollution circumstances in situ. Extrapolation from experimental laboratory conditions is unnecessary; the pollutants are acting on intact ecosystems. It has the equally distinct disadvantage of requiring destruction or alteration of an existing ecosystem in order to assess the toxicity of a pollutant. Consequently, while the field approach does permit some prediction based on extrapolation of observed ecosystem-pollutant interactions, this predictive ability is often obtained at high cost--namely, the alteration or loss of an existing ecosystem. Thus it would seem that ecological considerations per se, while providing important insight into the nature and extent of water pollution, are at present not readily modified to yield predictive bioassay measures.

The Laboratory Approach

While this category appears at first blush quite heterogeneous, laboratory researches in pollution have many common roots whether they be biochemically, physiologically, behaviorally, or histologically oriented. All are working with elements of whole organism form and function; all have removed the organism from its natural ecosystem to a drastically simplified one--the laboratory; conclusions obtained from any laboratory study require further assumptions and field experiment corroboration before they can be confidently extended to the ecosystem.

Attempts are currently being made to set up simplified ecosystems in the laboratory. The artificial streams developed by Doudoroff and Warren (personal communication) are an excellent example. These systems ideally permit the experimenter to control to some extent the many variables normally found in natural ecosystems, facilitating the manipulation of a single variable. However, even these ecologically oriented laboratory methods present serious extrapolative challenges.

It is abundantly clear that in recent years it has become progressively more difficult to separate the biochemical from the physiological, the physiological from the behavioral in biological research. Each level of study merges with the next; each contributes to and may become the cause or explanation of the other. The picture of "toxic effect" emerges clearly as a sequence or flow of biological events, beginning with a biochemical response of specific cells or cellular elements to a toxicant, radiating outward as those physiological and behavioral processes reliant upon the disturbed biochemical mechanism are thrown out of balance. This whole pattern is continually modified by the many homeostatic forces attempting to maintain the organism's internal equilibrium. The overall effect is transmitted to the ecosystem as changes in adaptiveness of the toxicated organism. Thus the altered nerve impulse transmission pattern resulting

from sublethal chlorinated insecticide toxication begins as a biochemical reaction at the cellular level, continues as a change in physiological responsiveness, and is transmitted to the ecosystem as aberrant behavior of the toxicated organisms.

There is therefore little doubt that the present dichotomy between the field and laboratory approaches, as between behavioral versus biochemical responses, is a temporary one. It is equally clear that the present diffuse and widespread controversy over the appropriateness of relating laboratory studies to field or ecological conditions is also a temporary one, and perhaps more apparent than real. For in reality, the biochemical change is incontrovertibly linked to the ecological change. Indeed, they are more than bound together; they are elements of a single phenomenon: the toxic response.

One obvious and immediate task of pollution biology is determination of the components and sequences of the many toxic responses produced by water pollutants. Achievement of this is dependent upon 1) recognition of the heterogeneity of these response components, and 2) definitive research on all aspects of the various components. For without this basic knowledge we are committed to courses of action which are at once empirical and blundering.

PROJECT ORIENTATION

The principal goal of the current Engineering-Science, Inc. research program is the laboratory screening of various parameters of the toxic response, with intensive study of those parameters which are readily quantifiable and seem amenable to formalization into standard bioassay procedures. An additional requisite is that the responses demonstrate toxicity at very low levels of toxicant concentration.

While it may be possible to conduct this screening on an entirely empirical basis--examining as many different aspects of form and function as can be readily conceived and measurement contrived--it seems more efficient to seek useful parameters of a specific toxic response among the various changes which occur when a toxicant and a living system come in contact. In some instances few or no steps in the sequence of action of a toxic response are known; in these cases empirical research is indicated. Generally, however, sufficient facts are at hand to construct at least a preliminary flow diagram of the toxic response, acknowledging all the while the hypothetical nature of many elements of the sequence.

Thus, when the organic pesticides were selected for study as important contemporary pollutants, several response parameters became immediately apparent. It has, for example, been reported in the literature that DDT is capable of action at numerous sites throughout the central nervous system (Beckman, 1961;p. 579). Other investigators have reported damage to liver and kidney tissues from the same and related compounds, suggesting several sites and modes of action. Integrated central nervous system patterns such as

conditioned responses are likely to be affected. Those aspects of metabolism involving detoxication and excretion of the toxicant are implicated. Thus the development of a toxic response flow pattern proved a useful method of selecting response parameters for evaluation. The technique has the important advantage of keeping the bioassays on the "main track" of meaningful biological changes occurring as direct responses to the toxicant's action. The possibility of misleading and spurious correlates is thereby reduced.

Limitations imposed by budget, availability of trained personnel, and time have further canalized the research orientation. Consequently, emphasis has been placed on physiological and behavioral measures, with the realization that important biochemical and ecological components of the toxic response have been neglected.

TOXICANT SELECTION

Organic insecticides have in recent years assumed national importance as agents of pollution. According to Nicholson (1960) during 1957 between 11,000,000 and 25,000,000 pounds of technical grade insecticide were applied to cotton fields in the six Southeastern states alone. Carson (1962) has reported overall organic pesticide production figures which indicate the growing importance of these poisons: a rise from 124,259,000 pounds produced in 1947 to 637,666,000 pounds in 1960.

The inevitable results of this ubiquitous and often indiscriminate use of these pesticides (or biocides, as they are sometimes more accurately called) are their appearance in water supplies and the resultant concentration in the tissues of exposed organisms. Both of these circumstances can and do grossly affect aquatic life. The U.S. Public Health Service (1961) reported the results of a survey, giving a highly conservative estimate of 5.6 million fish killed by "Agricultural Poisons" in 1961. The next most significant contributor to pollution-caused fish kills was "Industrial Wastes" responsible for reported kills totalling 2.9 million fish. The report was emphatic in its reservations about the applicability of the statistics. It stated (p. 1):

> "Such reports do not take into account the effects of adding to a stream small quantities of pollutants which may not kill fish but which still have a significant effect on the aquatic food organisms which support fish life. They do not take into account weak pollutants which inhibit fish growth and fish reproduction, nor the effects of pollutants which cause flesh tainting, rendering fish unpalatable for human consumption."

A considerable body of data is developing relative to the acute toxicity of the pesticides (e.g. Henderson, Pickering, and Tarzwell, 1959). However, there has been almost no work done on the chronic and/or sublethal effects of the pesticides to aquatic life. One notable exception to this is the recent work of Mount (1962) on blunt-nose minnows and guppies. There is little doubt that the

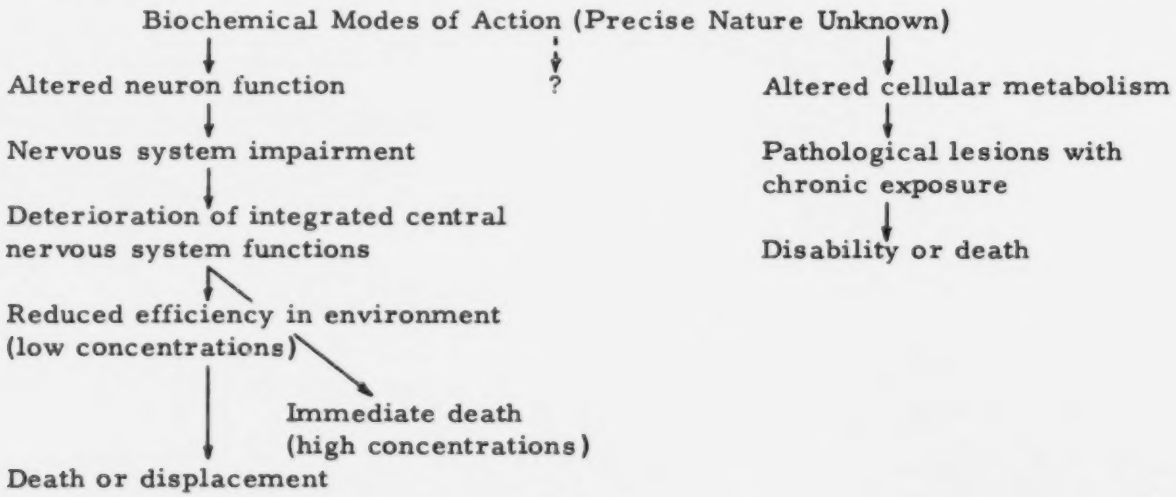
use of pesticides will continue, and in all liklihood the trend will be toward greater quantities applied over even larger areas than at present. New compounds of greater toxicity will appear on the market and unless more rigorous pre-release bioassay research is required than is now the case it is inevitable that public health groups will, as time progresses, be contending with more and more toxic compounds about which less and less is known.

In summary, the many reports of organic insecticide residues appearing in natural waters, the dearth of knowledge relative to their modes of action and toxicity, and their demonstrated profound effect on our natural resources led to the selection of organic insecticides as experimental toxicants.

RESPONSE PARAMETER SELECTION

Recent work in several laboratories indicates that a wide variety of responses may lend themselves to quantification. Significant progress in this area was recently made by Klock and Pearson (1961). In this instance one principal criterion was that the responses reflect changes in metabolic activity and be readily quantified. It is unfortunate that these workers used a non-parametric mathematical approach to the analysis of their data, which provided a "normalized relative response rate" curve for each toxic response. This approach, while novel and ingenious, is unfamiliar to most biologists and has rendered their findings less available for evaluation and study.

It is now clear from the work of several investigators that the organophosphates are distinctly different in their toxic action from the chlorinated hydrocarbons. Albert (1960), among others, recognizes that the site of action of the chlorinated hydrocarbon insecticides is on nerve ganglia, but that the effect is still imperfectly understood. Even within the chlorinated insecticides, while BHC, Dieldrin, Aldrin, and Endrin all seem to act by a similar mechanism, DDT has a physiological action which is unique despite its chemical alliance with the other chlorinated hydrocarbons. Other workers have demonstrated several discrete sites of pathology in Toxaphene (a chlorinated camphene) poisoning. The toxic response sequence in this case is believed to be:


Biochemical Modes of Action (Precise Nature Unknown)
Altered neuron function
?
Altered cellular metabolism
Nervous system impairment
Pathological lesions with chronic exposure
Deterioration of integrated central nervous system functions
Disability or death
Reduced efficiency in environment (low concentrations)
Immediate death (high concentrations)
Death or displacement

Hence it was concluded that objective measures of nervous system function offered one productive avenue for quantifying sublethal toxic effects. Quantifiable growth processes such as regeneration, appeared suitable as indicators of metabolic impairment.

The Conditioned Response

The conditioned response is in many ways an ideal function to study in behavioral pathology. Development of a conditioned response is a form of learning which is ubiquitous in the animal kingdom--everything from protozoans and coelenterates to mammals have been demonstrated to possess the ability. Cason (1925) has an excellent discussion of the universality of the response in living organisms. However, it must be kept in mind that the response, though behaviorally similar in different forms, may be achieved through quite different processes in the different phyla. The elaborate nervous system of the mammal would not, for example, be available to the amoeba as a means of data storage and integration, yet the response can be readily elicited in both forms. In all probability analagous conditioned response systems have been evolved in the different phyletic groups.

The process of conditioning is used extensively by living things, and becomes considerably refined in the higher vertebrates. Unfortunately, study of this and other forms of learning in aquatic organisms has been large neglected over the years, leaving us with only a suggestion of its true magnitude in nature. Noble and Curtis (1939) demonstrated that color preference (hence proper identification of parent; and later, mate) in young jewel fish was very largely learned. Their studies indicated that conditioning in early life can affect the response to colors in several species of cichlids. Seitz (1940) found that simple models did not induce courting in the male Tilapia. Apparently there is a very detailed and presumably conditioned (learned) knowledge of the female characters. Tilapia young have markings very similar to the color pattern of the ripe female. It is possible that this learning process takes place in the schools of immature fishes.

Herter (1953) has clearly demonstrated the wide range of learning ability in fish, including discrimination of quite unnatural forms (to the fish) as squares and triangles. A number of workers have examined the unique capacity of salmon and other anadromous fish to learn and remember the characters of their early environment well enough to find it again upon their return several years later. Rich and Holmes (1928) were among the first to transplant salmon eggs from the spawning grounds to new streams. The salmon, when mature, returned to the river where they were raised rather than where spawned. Shapovalov (1940) found that over a four year period, 97.9 per cent of steel-head trout (Salmo gairdneri) returned to the home stream and 2.1 per cent to a stream whose mouth was only four miles distant. Hasler and Wisby (1951), in an ingenious series of experiments, demonstrated that salmonids learned the olfactory nature of the home creek's waters.

These and other recent studies have conclusively shown that anadromous fish (salmon, striped bass, steel-head, etc.) have an extraordinary capacity to learn the nature of their early surroundings, remember it for up to five years, and then apply the remembered data to the problem of locating the home stream. Thorpe (1956) in his book, Learning and Instinct in Animals, comments that the homing performance of salmonids "...is very like learning to run a complex maze in reverse as a result of one prolonged experience of the maze. It is not necessary to assume that the fish remembers every detail of the hundreds of miles which its journey may cover, but one must suppose that it remembers the characteristics of the different sections and particularly the features of the various 'choice points' constituted by the junctions of the tributary streams with the main river".

It has been pointed out that one of the principal modes of action of the organic insecticides is via nervous system dysfunction. That this dysfunction should contribute to impaired conditioned responses and other forms of learning is an expected corollary. It has recently been ascertained that other workers are finding the conditioned response a useful tool in quantifying drug and other effects. Aston, Sekino, and Greifenstein (1962) are using conditioned avoidance behavior in the rat to quantify drug effects on that species. Scarborough and Addison (1962), in exploring the effects of x-irradiation on fish, found that the acquisition of conditioned responses was suppressed at sublethal dose levels. Preliminary experiments, reported beyond, have established that there are indeed changes in learning patterns in fishes with organic pesticide toxication. These changes in learning ability and performance are objectively measureable. With sufficient refinement of procedure, they will permit rather precise quantification of degree of sublethal toxication.

It should be emphasized that while loss of the ability to develop a conditioned response may be detrimental to the animal per se, this measure of the ability of an organism to produce integrated neuro-muscular responses additionally provides considerable insight into the general condition of toxicated organisms.

Regeneration

Regeneration is the regrowth of lost body parts. It is utilized as a reproductive mechanism in many lower invertebrates, where fragmentation of adult animals is followed by regeneration of the pieces into completely formed and functional offspring. As a mechanism of repair and replacement of lost body parts regeneration is found ubiquitously throughout the animal kingdom, being especially prominent and important up through the amphibia. In common with the more primitive groups, amphibians can--by regeneration--replace whole structures which have in some way been lost.

There are numerous instances in the literature of the rate and pattern of regeneration being used as a measure of toxicity, for example the work of Quaglio, et al. (1957) on the toxin holothurin. Properly used, this technique can

provide useful information on metabolic poisons. In addition, work by Singer (1952), Schotte and Harland (1943), Kamrin and Singer (1955) and others has clearly defined a regeneration-nervous system relationship wherein interruption of the normal function of adjacent nervous tissue inhibits the regeneration process.

Of equal significance is the interruption of regeneration which has been demonstrated by substances known to interfere with the acetylcholine mechanism of the nerve synapses (Singer, 1954; Singer, et al., 1955). Since the organophosphates suppress the cholinesterase level (the enzyme which inactivates acetylcholine after it has been liberated at the nerve synapses), toxication by a compound like Parathion (an organophosphate) leads to excessive concentrations of acetylcholine in the nervous tissue. The effect of this on regeneration should be examined. It is the project staff's opinion that any toxicant materially interfering with the normal regenerative processes of aquatic organisms could have profound ecological consequences.

Total Activity

"Total activity" may be thought of as the sum of the organism's overt actions or behavior; its movements, gestures, paddling and finning, etc. Significant relations between activity and age, temperature, illumination, sex, periodicity of feeding, and other conditions have been demonstrated through this measure (e.g. Young, 1936).

Reports have been received (Dr. Donald Mount, personal communication; F.M. Atton, personal communication) that chronic exposure to chlorinated hydrocarbons increases the responsiveness of fish to external stimuli. This hypersensitivity to external stimuli and "restlessness" have been observed in pesticide-toxicated fishes at this laboratory as well. A measure of the total activity would permit accurate quantification of this change in behavior.

It is realized that without related studies to clarify the significance of changes in total activity, the measure is of only nominal usefulness. In this instance, the situation bears considerable similarities to the work of Weiss (1958;1959) who first demonstrated the striking suppression in fish brain cholinesterase level following exposure to the organophosphates. His findings have been criticized by some as being "inapplicable" to ecological interpretation, and therefore useless from the standpoint of a bioassay procedure. This is possibly so today; but the only thing required to greatly enhance the "usefulness" of Weiss' findings is sufficient insight into other aspects of the organophosphate toxic response. Once this insight is attained, and the sequence of organophosphate toxication more clearly understood, Weiss' method may well become the standard bioassay for this class of poisons. Similarly, total activity, while in itself of perhaps limited usefulness, when combined with other parameters of the toxic response may provide useful insights. For example, correlation of

total activity, hypersensitivity to external stimuli, and conditioned response acquisition may yield a description more directly applicable to ecological circumstances.

Coelenterate Behavior

Certain coelenterates, for example the hydra (Pelmatohydra oligactis), are readily maintained in laboratory cultures. It was concluded that use of a coelenterate might be advantageous, from both the standpoints of ease of handling and the possibility of providing needed data on the toxic responses of primitive invertebrates.

Feeding behavior, responses to tactile and other external stimuli, and general pattern of behavior when undisturbed are all quantifiable to some degree. These parameters have to date proved the least productive of the several examined.

III. APPARATUS

SERIAL DILUTION UNIT

To maintain the desired environmental circumstances for long-term behavioral and physiological studies at sublethal toxicant concentrations, a dependable continuous-flow apparatus is necessary. A thorough study of the literature (e. g. The Staff of the Water Pollution Research Laboratory, 1951) and of existing commercially available devices indicated that nothing suitable was available at less than prohibitive cost. It appeared, in addition, that such an apparatus, if properly designed and of nominal cost, might prove of utility to pollution researchers and others--such as the pesticide industry--who are interested in quantification of toxicity to aquatic organisms.

A continuous-flow serial dilution system, capable of delivering a wide range of toxicant concentrations over long periods of time with minimum maintenance and adjustment, was therefore designed and constructed. Since field studies of the effects of toxicants were also contemplated, the apparatus was made as self-sufficient as possible. To preclude the necessity of frequent chemical analyses the delivered flow rates of toxicant solutions were set relatively high, thereby avoiding absorption, adsorption, and degradation difficulties with their concomitant biases to experimental results.

Figure 1 illustrates the relationship of the serial dilution unit to the other bioassay components. It will be noted that the fresh water source is derived from the municipal water main and is filtered through a 5μ cellulose filter before entering the water storage tank. The aerated water storage tank combines

the characteristics of a constant head device and dechlorinator, being of sufficient size to provide a nominal 24 hour retention period. A flow meter in the line connecting the water storage tank to the serial dilution unit provides a visual means of monitoring input into the serial dilution unit. As will be seen, the rate of cycling of the serial dilution unit and the output of the eight delivery cells is controlled by this flow into the dilution unit. Thus, for a dispensing rate of 200 ml/min at each concentration, the flow meter is set at 1600 ml/min.

Concentrated toxicant is added to the serial dilution unit via a plunger-type automatic pipette. The one presently utilized is manufactured by Misco, Inc. All contact parts of the pipette are of glass, permitting the use of acetone as a solvent and vehicle. The pipette is mounted at the side of the unit and actuated intermittently by a weighted float. Toxicant flow rates from eight delivery cells can be varied from approximately 30 ml/min to 400 ml/min as desired.

The arrangement of aquaria in the constant temperature tank as indicated in Figure 1 was the setup used in one type of experiment. An alternate layout, and the one used in subsequent studies, utilizes one large aquarium for each toxicant concentration. In these large aquaria are placed cages of fine mesh nylon net containing the various groups of test organisms. This latter arrangement avoids many of the complexities and variables encountered when each organism or species is maintained independently from the rest.

Figure 2 presents a more detailed diagrammatic representation of the serial dilution unit. Some of the steps in a cycle of the unit are illustrated in the individual cells although in practice the moving parts of each cell act simultaneously.

With reference to Figure 2 a brief description of the operation of the serial dilution unit through one cycle is as follows:

1. Collecting tank fills at a fixed rate from the constant head water storage tank.

2. When the appropriate volume is reached, the collecting tank empties by way of the automatic manifold siphon. The water passes through the distribution manifold and hence into the eight delivery cells.

3. Lowering of the water level in the collecting tank actuates the toxicant injector through lowering of the weighted float; the appropriate amount of toxicant is injected into delivery cell A.

4. Toxicant and influent fresh water are mixed both by the jet effect of the distribution manifold and the air diffuser, each delivery cell being equipped with a diffuser as shown in cell H. It will be noted that during this period of filling of the delivery cells, the delivery siphons are inactive. This inactivation resulted when the delivery cells were emptied at the end of the previous cycle.

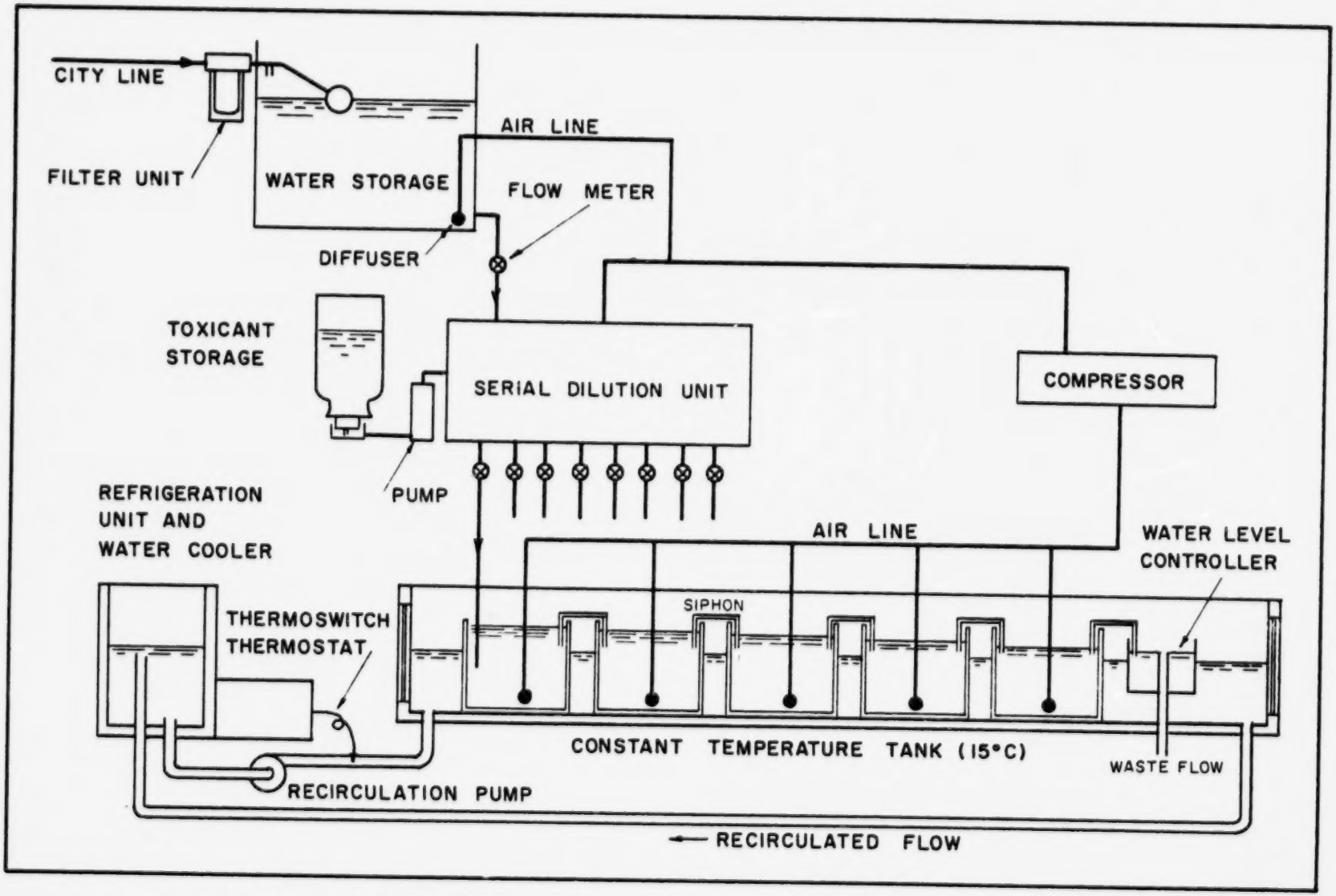


FIGURE I. DIAGRAMMATIC REPRESENTATION OF BIOASSAY EQUIPMENT

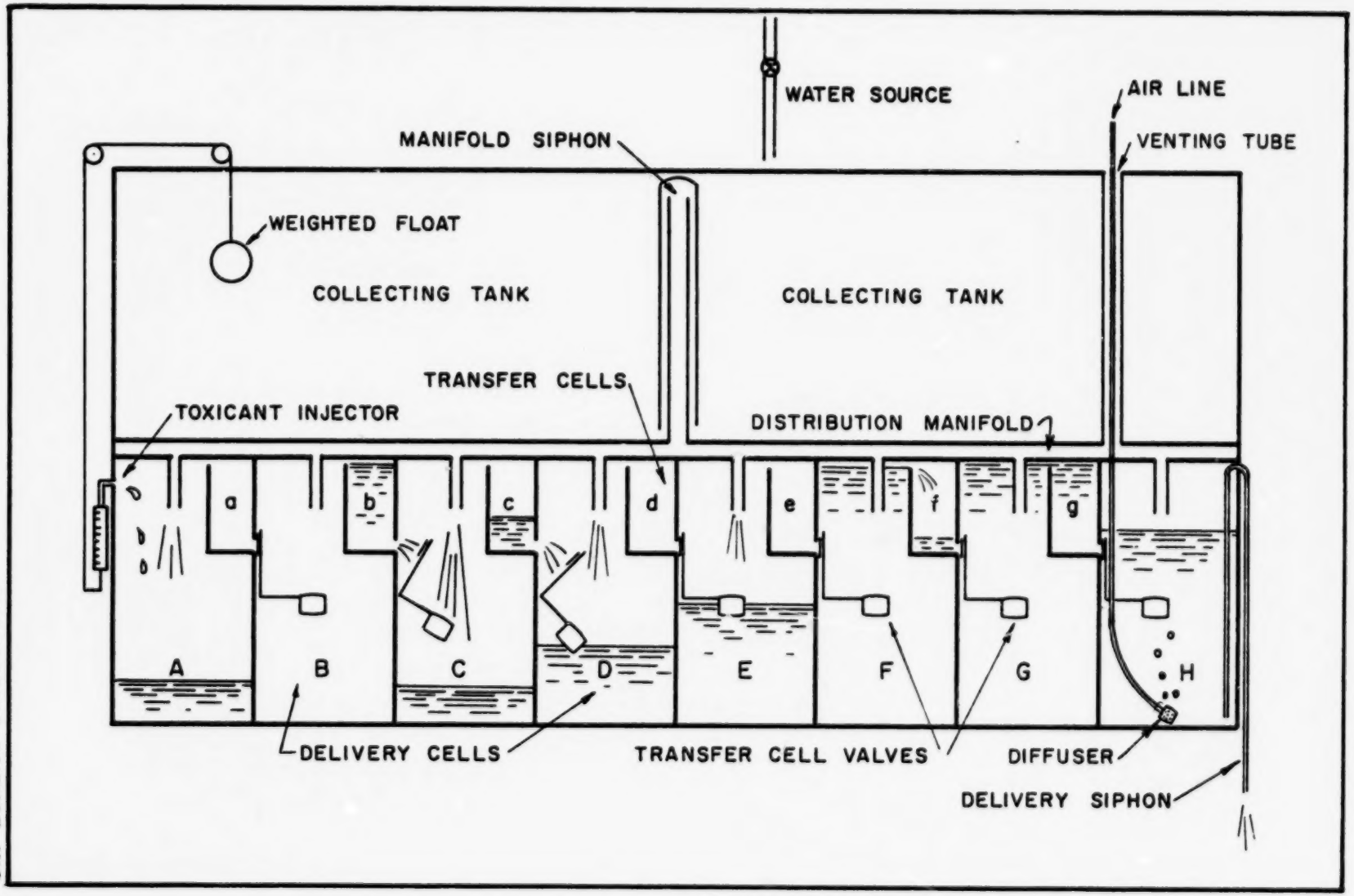


FIGURE 2. DIAGRAMMATIC REPRESENTATION OF SERIAL DILUTION UNIT

5. Water entering the delivery cells from the distribution manifold opens the transfer cell valves as shown in cell C. Contents of the transfer cells empty into the adjacent delivery cells as in cells b to C, and c to D.

6. The delivery cells fill with toxicant and water, closing the transfer cell float valves in the process. Delivery cells E and F illustrate this condition. After the delivery cells have filled and before the collecting tank empties, the transfer cells are filled by overflow from the delivery cells as shown in cell F to f; the final completely filled condition is shown in cell G.

7. As a consequence of the completed filling of delivery cells, the delivery siphons are activated and toxicant flow to the test unit is reinstituted.

8. The delivery cells all drain simultaneously by way of the delivery siphons. The transfer cell valves remain closed during this draining, being held in place by small magnetic locks.

9. It will be noted that the unit must cycle nine or more times when a new toxicant is introduced before all delivery cells become functional.

The body of the serial dilution unit was constructed of 1/4" and 1/8" translucent rigid polyvinyl chloride (PVC) plastic. Other components of the apparatus are of stainless steel, neoprene rubber, and expanded polystyrene plastic. None of these products have any known toxic qualities.

Figures 3 through 6 illustrate certain components of the unit, and the unit as it appears when in operation. Figure 7 illustrates the volume-flow relationships when the unit is in operation. It should be emphasized that although the unit and the volume-flow relationships may at first appear complex, actual operation of the unit is exceedingly simple. The unit is installed near the test aquaria, and a water supply is provided from whatever source is desired. Either salt or fresh water may be used. The injector pipette is set to deliver the desired quantity of toxicant and the water turned on. No electrical hookups are required, all functions of the dilution unit being accomplished by the kinetic energy of the influent water plus the low pressure air used for mixing and maintaining the water in a well-aerated state. The unit illustrated in Figure 3 has been in operation continuously for five months with no down time for adjustment or repair.

CONDITIONED RESPONSE APPARATUS

Apparatus capable of developing a simple conditioned response in aquatic organisms was designed, constructed, and tested in behavior experiments. While primarily designed for fish, it can be used with minor modifications for a wide variety of aquatic forms. Figure 8 illustrates the essential circuitry and layout.

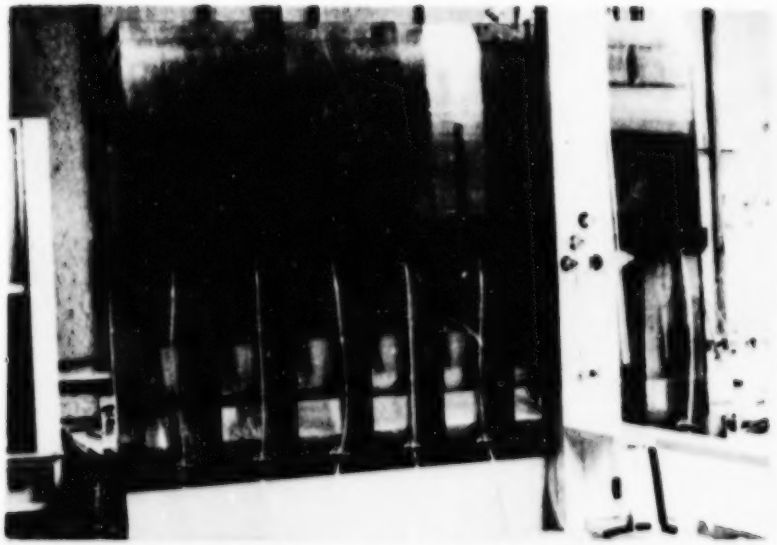


FIG. 3. SERIAL DILUTION UNIT IN OPERATION
Delivery Siphon tubes in foreground

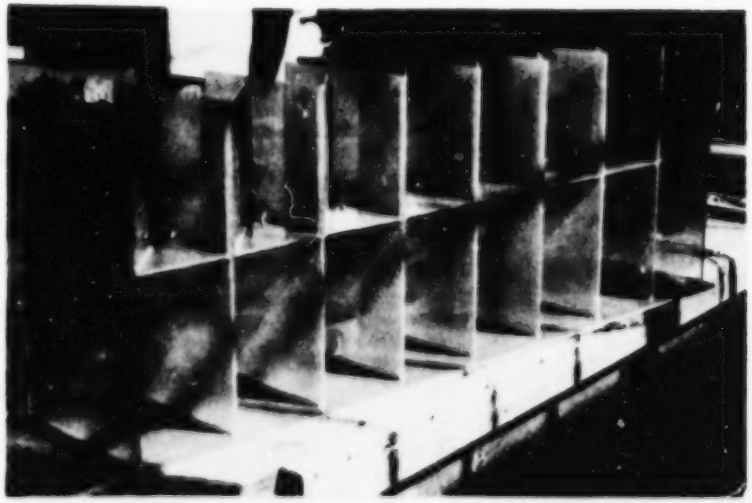


FIG. 4. SERIAL DILUTION UNIT UNDER CONSTRUCTION

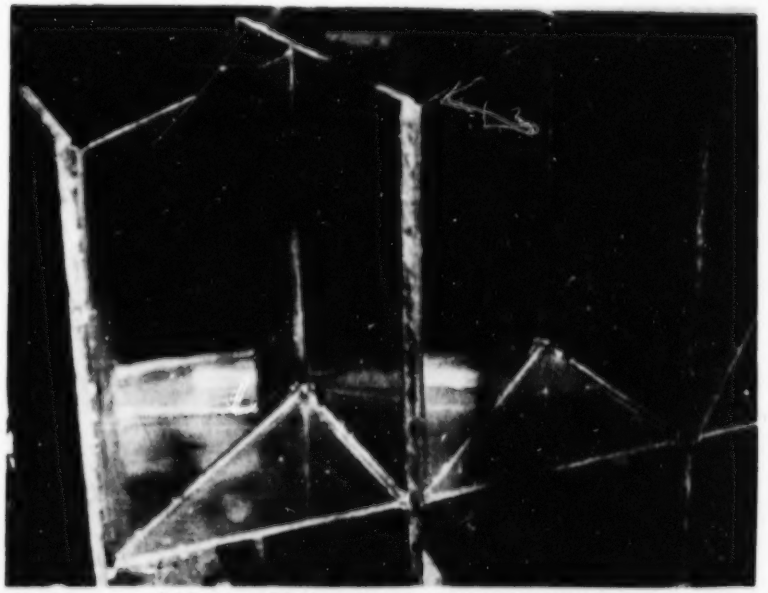


FIG. 5. DETAIL OF TRANSFER CELLS
Note drain holes

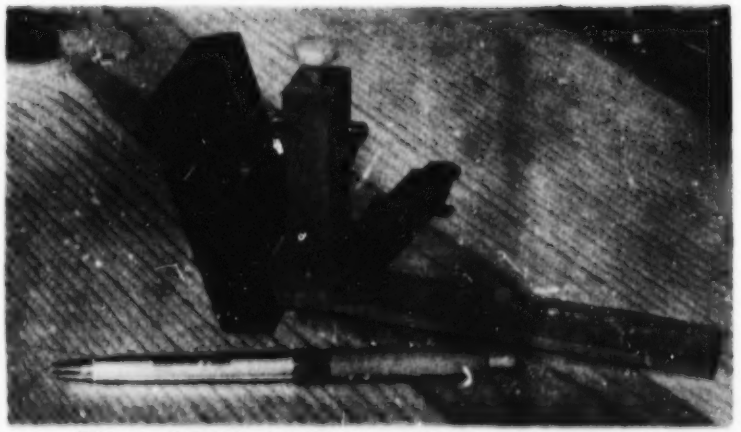


FIG. 6. DETAIL OF TRANSFER CELL VALVE WITH MAGNET LOCK.. Float cup removed. Through bolt is magnetic stainless steel.

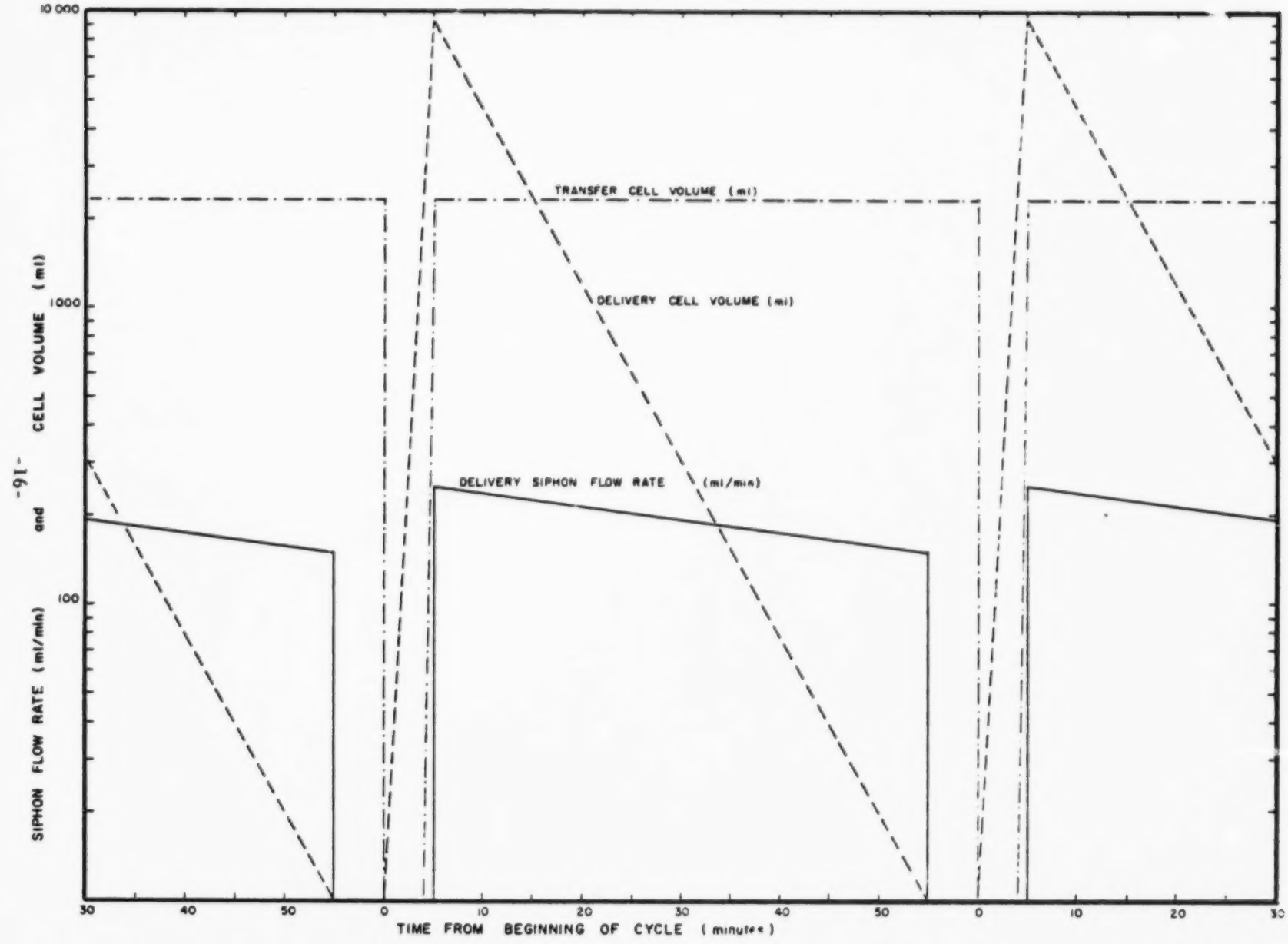


FIG. 7 SERIAL DILUTION UNIT VOLUME-FLOW RELATIONSHIPS THROUGH TWO CYCLES

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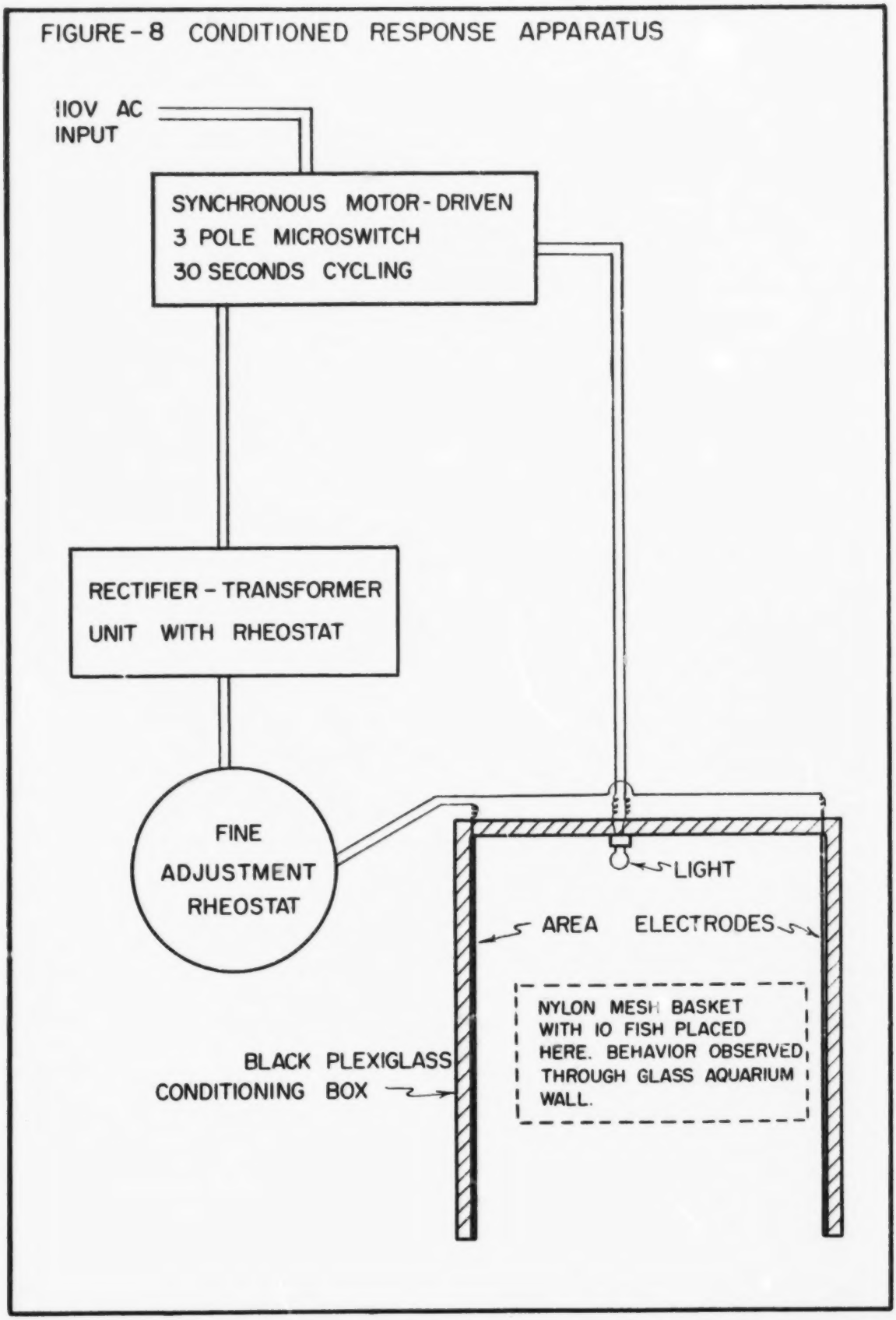
FIGURE-8 CONDITIONED RESPONSE APPARATUS



The rheostats are adjusted to create a voltage drop of 10-14 V across the water volume encompassed by the electrodes. Nylon mesh baskets, each holding 10 experimental fish, are placed in the conditioning box, and the synchro-motor micro-switch system activated.

The conditioning cycle which in the experiments is referred to as one trial is as follows:

1. 25 seconds with both light and shock off, followed by

2. 3 seconds of light only, followed by

3. 2 seconds of light + shock.

The shock intensity is adjusted so as to be disturbing to the fish (i.e. elicits escape movements) but not damaging. The current is transformed and rectified to one-half wave pulsating direct, 60 cycles per second. It has been determined through experimentation and literature study (e.g. Scarborough and Addison, 1962) that this type of current produces an optimal conditioning effect.

A positive conditioned response induced by this apparatus is defined as any abrupt change in behavior at or after the moment of onset of light, but before the shock is delivered. Such responses include rapid sinking, backing up, darting ahead, agitated fanning or fluttering of pectoral fins, and attacking another fish. Often individuals are observed to stop whatever they are doing, hover motionless for one or two seconds, and then proceed with the aversive behavior, all before the onset of shock. Scores for these trials are the number of individuals within the group showing aversive behavior during the "light only" interval of the conditioning cycle. All conditioning tests are made with the aquarium enclosed in an opaque box equipped with a panel of one-way glass, through which observations are made.

CONDITIONED AVOIDANCE RESPONSE APPARATUS

Further study of conditioned responses, and of the need to relate bioassay measures as closely as possible to ecological equivalents led to the design of a more complex conditioned response unit. It was felt that:

1. The previously described unit left undue room for subjective interpretation by the observer, despite the apparently successful use of somewhat similar devices by other experimenters (e.g. Scarborough and Addison, 1962).

2. Scientists unfamiliar with the theoretical aspects of the conditioned response might reject the measure as lacking in applicability.

3. The use of 10 anonymous fish per group substantially reduces the data obtained from a single test.

4. Other behavioral phenomena can be successfully quantified simultaneously with an appropriately designed unit, permitting a more efficient gathering of data.

Consequently, an improved unit, capable of producing a conditioned avoidance response was designed and constructed. This unit provides an escape or sanctuary area for the test animals, permitting them to flee from the area receiving electric shock to a "safe" region. It can be seen that such a design more nearly approximates the natural situation, the animal learning the location of refuges or areas of safety when exposed to adverse stimuli. With unavoidable shock, the integrative process of "escape" is not elicited.

The essential elements of the unit together with the pertinent circuitry are illustrated in Figure 9. It will be noted that the "safe" area is that half of the apparatus which is inactivated, i. e. not receiving light or light + shock. Tests using this unit have satisfactorily demonstrated that 1) fishes have the ability to learn conditioned avoidance responses; 2) toxicants can impair this ability; and 3) other important data on toxicated behavior can be simultaneously obtained.

An advanced design of this unit is presently under construction. It will utilize the same principles, but will have dividers separating individual fish. This will permit determination of within group differences and allow statistically powerful analyses. It will additionally prevent the possibility of "follow the leader" or pseudo-learning responses. Another feature of the unit under construction is an automatic response recording system. Advantages are greatly improved precision in data gathering and simultaneous quantification of several other elements of the avoidance response. The unit is designed to permit testing of experimental organisms while still in the toxicant solutions of the various test aquaria.

TOTAL ACTIVITY COUNTER

A unit measuring the total activity of experimental animals has been designed, constructed, and tested. The pertinent circuitry and layout are illustrated in Figure 10. The light source and sensing unit utilized in the total activity counter were manufactured by C. H. Electronics, Inc. of South Gate, California. The unit is actuated by one of the test organisms interrupting the light beam passing through the test chamber, the incident being recorded on an electromagnetic counter. Striking differences in the total activity of experimental and control groups during the preliminary trials were recorded; sixteen-fold increases recorded on one run. The apparatus is presently being modified to improve its sensitivity. This will allow faster processing of test groups. The overall outlook for this technique is most encouraging.

FIGURE - 9 CONDITIONED AVOIDANCE RESPONSE APPARATUS

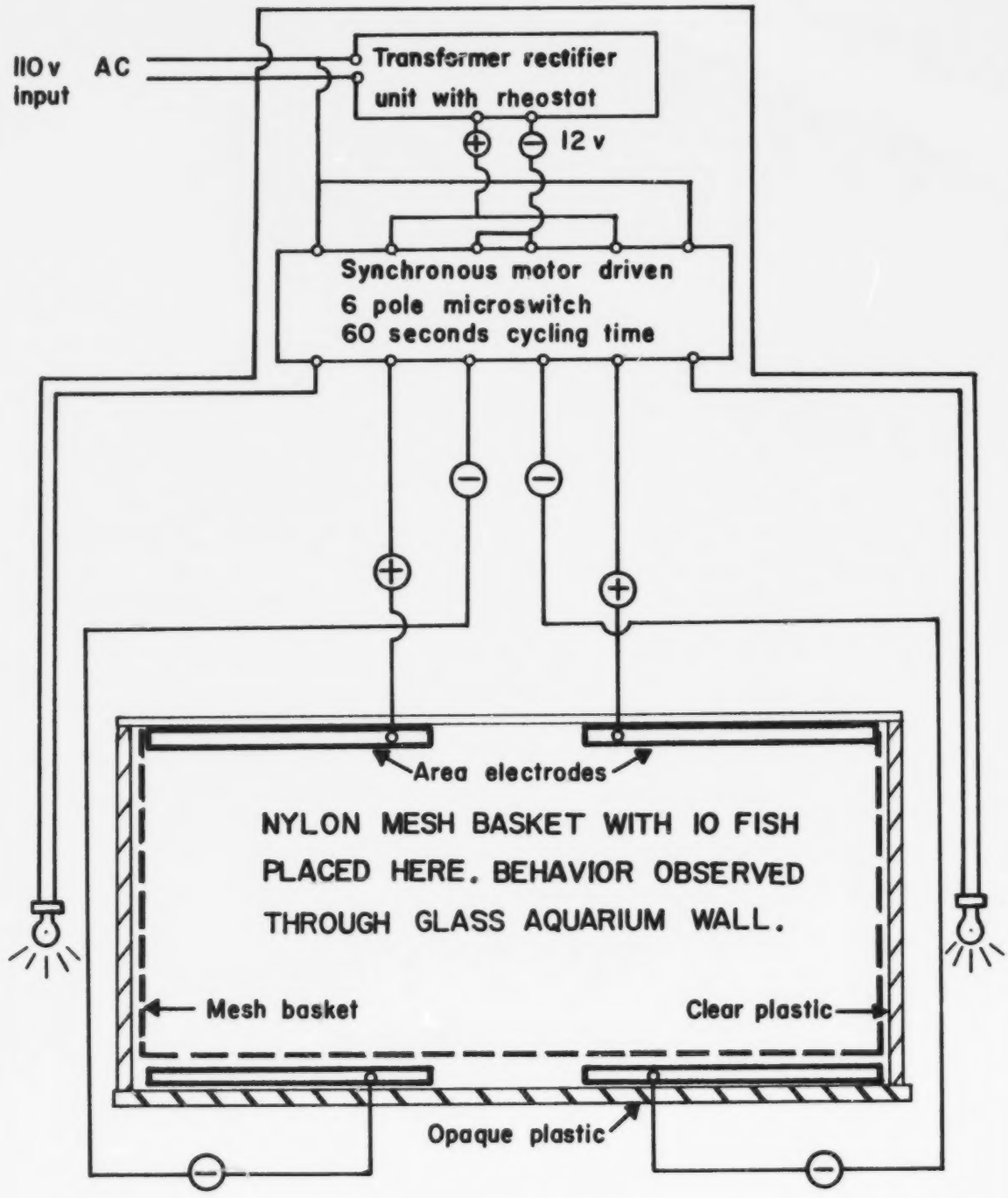
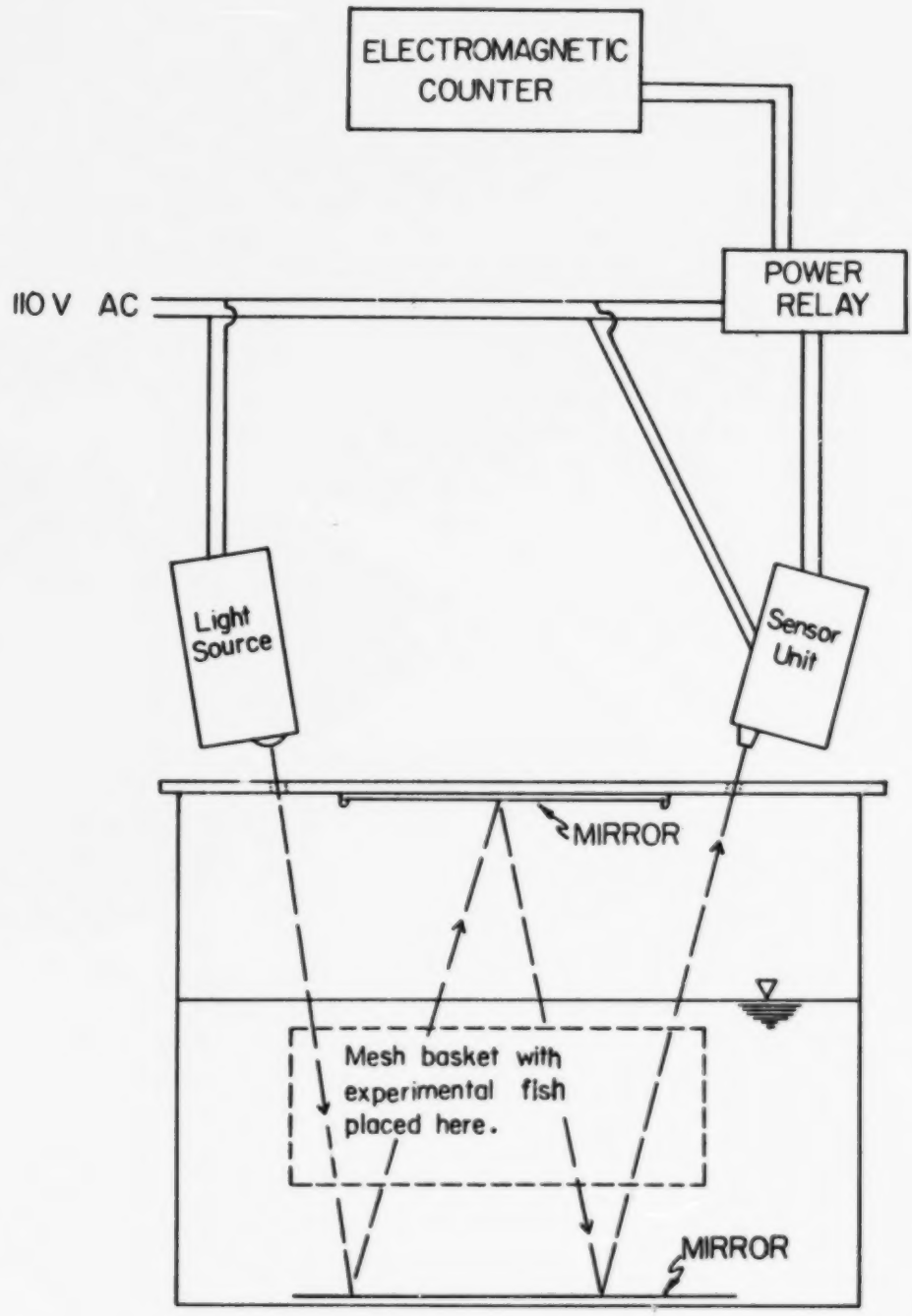


FIGURE - 10 TOTAL ACTIVITY COUNTER



IV. EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENT M-1. EFFECTS OF 24 HOURS EXPOSURE TO TOXAPHENE FOLLOWED BY 24 HOURS IN FRESH WATER UPON A SIMPLE CONDITIONED RESPONSE IN THE MOSQUITO FISH, GAMBUSIA AFFINIS.

Introduction

There are many ways of utilizing experimentally the conditioned response. For example, Bull (1957) has done extensive work on the discrimination abilities of fishes using the conditioned response as an indicator of stimulus detection. In this and the following experiments the subjects were first toxicated, and the conditioned response acquisition patterns recorded. Another procedure consists of conditioning subjects, toxicating them, and subsequently measuring retention of the conditioned response. The latter procedure will also be used in later experiments.

Materials and Methods

Test Organisms

Mosquito fish (Gambusia affinis) were obtained by dip netting from a small reservoir on the Jack London Ranch in Napa County, California. Interview of the property owners revealed that no insecticides had been used on the watershed of this lake; the fish were therefore concluded to be free of significant previous exposure to organic pesticides. The fish were placed in temperature-controlled, aerated aquaria and held one week for observation and acclimatization to laboratory conditions.

Toxicant System

The serial dilution unit and temperature-controlled toxicant system previously described were used. Temperature was held at 18± 0.5°C. Toxaphene concentrations in the toxicant tanks were as follows:

T/1	=	1.0 Mg/L	(1 ppm)
T/2	=	0.2 Mg/L	(0.2 ppm)
T/3	=	0.04 Mg/L	(0.04 ppm)
T/4	=	8×10^{-3} Mg/L	(8 ppb)
T/5	=	1.6×10^{-3} Mg/L	(1.6 ppb)
T/6	=	3.2×10^{-4} Mg/L	(0.32 ppb)
T/7	=	6.4×10^{-5} Mg/L	(64 ppt)
T/8	=	1.3×10^{-5} Mg/L	(13 ppt)

The toxicant used was a 90 per cent solution of technical grade Toxaphene, a

chlorinated camphene, obtained through the courtesy of California Chemical Company, Richmond, California. The Toxaphene was manufactured by the Hercules Powder Co., Wilmington, Delaware. On the basis of volume-concentration relationships of the serial dilution unit, it was calculated that with the injector pipette set to deliver 2.0 ml of toxicant, a solution of 6.358 gm of 90 per cent Toxaphene/liter of acetone would provide a T/1 (toxicant tank Number 1) concentration of 1.0 Mg/L Toxaphene. Acetone was used as the vehicle. Previous investigation (Henderson, Pickering, and Tarzwell, 1960) has demonstrated that acetone is minimally toxic to fish.

Toxication Procedures

A total of 20 groups (8 experimental, 2 control, 1 replication) of fish were made by randomly distributing fish of approximately 2.5 cm length among 10 nylon mesh baskets. The groups of fish were then placed in the toxicant tanks for 24 hours, after which time they were removed to fresh uncontaminated water for 24 hours before testing in the conditioned response apparatus.

Testing Procedures

The simple conditioned response apparatus described in Figure 8 was used. The group of fish to be tested was transferred from the toxicant solution to a 10 gallon aquarium filled with control water at 18.0°C, by moving the nylon mesh basket containing the fish from one tank to the other.

The synchro-motor micro-switch unit was then switched on, beginning the conditioning cycle. Observations and scoring of responses followed, as described on page 18 of this report.

The behavior of the fishes was observed through the glass wall of the aquarium, positive responses being recorded by the observer.

Results

Table 1 indicates the general condition of the test and control groups during the pre-test exposure period.

Figure 11 illustrates the conditioned response patterns at two levels as compared to a control group. In this and the following graphs one trial equals one cycle of light followed by light + shock as described on page 18. It will be noted that the toxicated fish in this experiment were given 24 hours to recover in fresh water before being tested. Despite this recovery period, significant differences in behavior patterns were evident.

Figure 12 presents the response data in a different fashion. Here, the total number of positive responses by fish exposed to a given toxicant concentration are plotted as 1) the first 25 trial subtotal; 2) the second 25 trial subtotal; and 3) the overall total for the experiment. It can be seen that with decreased

TABLE 1

EXPERIMENT M-1. GENERAL RESPONSE OF MINNOWS TO TOXAPHENE

CONCENTRATION	TOTAL HOURS OF EXPOSURE	
	15 Hours	24 Hours
Control 1	Normal	Normal
Control 2	Normal	Normal
1 Mg/L	All dead	-------
0.2 Mg/L	8 dead	All dead
4×10^{-2} Mg/L	All alive; hyperactive, agitated.	As 15 hours
8×10^{-3} Mg/L	All alive; hyperactive.	As 15 hours
1.6×10^{-3} Mg/L	All alive and normal appearing	Hyperactive
3.2×10^{-4} Mg/L	All alive and normal appearing	Hyperactive
6.4×10^{-5} Mg/L	All alive and normal appearing	Hyperactive
1.3×10^{-5} Mg/L	All alive and normal appearing	Hyperactive

Note that the 24 hour TL_m for this experiment was between 0.2 and 0.4 Mg/L (0.2 and 0.04 ppm).

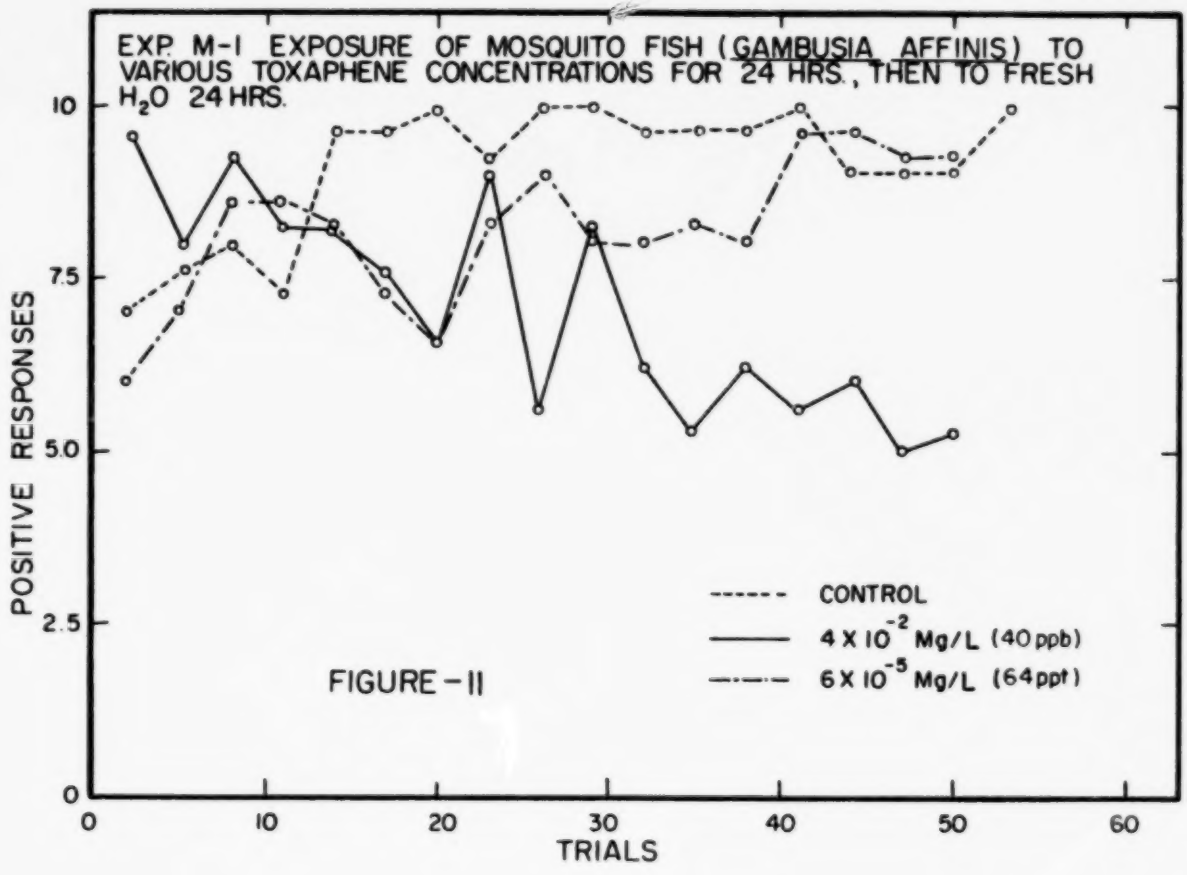

EXP. M-1 EXPOSURE OF MOSQUITO FISH (GAMBUSIA AFFINIS) TO VARIOUS TOXAPHENE CONCENTRATIONS FOR 24 HRS., THEN TO FRESH H_2O 24 HRS.
POSITIVE RESPONSES
10
7.5
5.0
2.5
0
0
10
20
30
40
50
60
TRIALS
CONTROL
4×10^{-2} Mg/L (40 ppb)
6×10^{-5} Mg/L (64 ppt)

FIGURE - 11

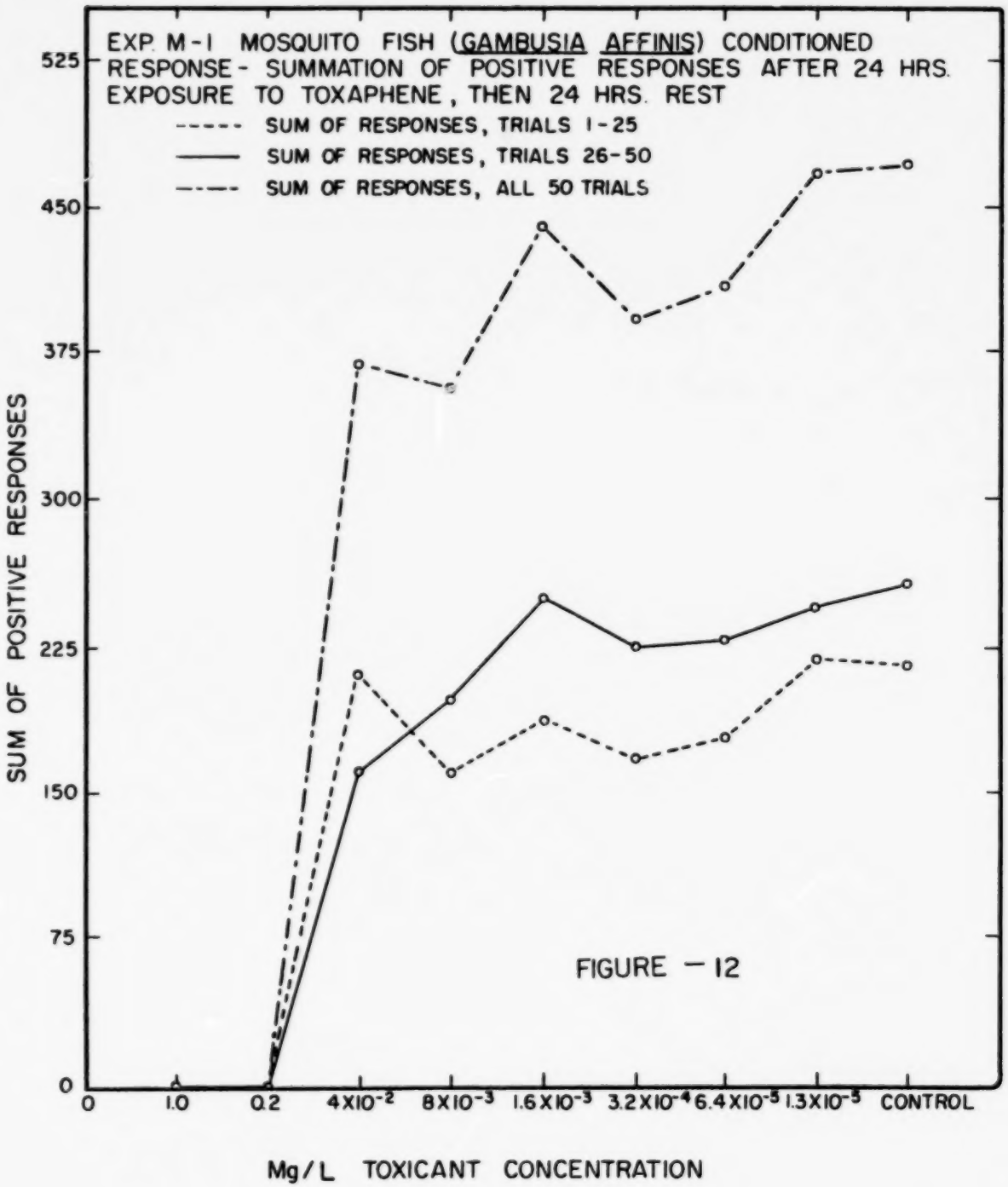
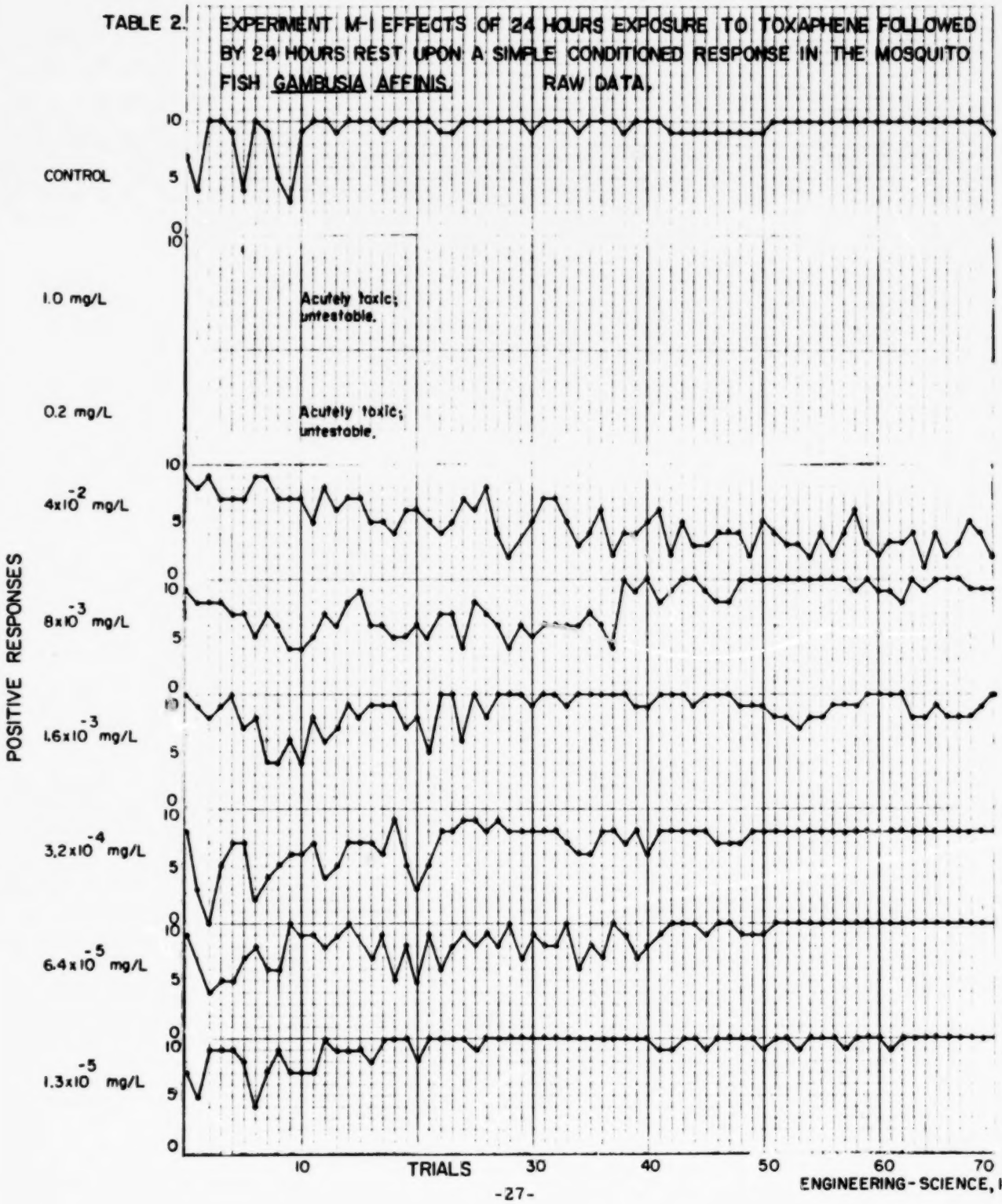

EXP. M-1 MOSQUITO FISH (GAMBUSIA AFFINIS) CONDITIONED RESPONSE - SUMMATION OF POSITIVE RESPONSES AFTER 24 HRS. EXPOSURE TO TOXAPHENE, THEN 24 HRS. REST
SUM OF RESPONSES, TRIALS 1-25
SUM OF RESPONSES, TRIALS 26-50
SUM OF RESPONSES, ALL 50 TRIALS
SUM OF POSITIVE RESPONSES
525
450
375
300
225
150
75
0
0
1.0
0.2
4X10-2
8X10-3
1.6X10-3
3.2X10-4
6.4X10-5
1.3X10-5
CONTROL
Mg/L TOXICANT CONCENTRATION

FIGURE – 12

TABLE 2. EXPERIMENT M-1 EFFECTS OF 24 HOURS EXPOSURE TO TOXAPHENE FOLLOWED BY 24 HOURS REST UPON A SIMPLE CONDITIONED RESPONSE IN THE MOSQUITO FISH GAMBUSIA AFFINIS. RAW DATA.



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toxicant concentrations, the overall performance is higher.

EXPERIMENT G-1.1. EFFECTS OF 24 HOURS EXPOSURE TO TOXAPHENE UPON A SIMPLE CONDITIONED RESPONSE IN THE GOLDFISH CARASSIUS AURATUS.

Materials and Methods

Test Organisms

Goldfish (Carassius auratus) of the "comet" strain were purchased from the Hayward Goldfish Farm, Hayward, California. Interview with the owner of this concern indicated that he did not use pesticides or herbicides around the fish ponds, limiting his use of chemicals to copper sulfate and sodium chloride in treating disease conditions. While it is possible that these fish had experienced previous exposure to insecticides the probability of such an occurrence was concluded to be minimal.

The fish were placed in temperature-controlled (18± 0.5°C) aerated aquaria and permitted to acclimatize for one week.

Toxicant System

The same toxicant system was used as described in Experiment M-1. Toxaphene concentrations in the toxicant tanks were as in Experiment M-1.

Toxication Procedure

Ten groups were made by selecting fish of approximately 4 cm length and randomly distributing them into 10 nylon mesh baskets. The baskets of fish were then placed in the toxicant tanks containing graded concentrations of Toxaphene. Time of introduction of the test groups to the toxicant was staggered so as to permit testing at 24 hours total exposure time.

Testing Procedures

The testing procedure was the same as that employed in Experiment M-1, and as described on page 18 of this report.

Results

Table 3 indicates the general condition of control and test groups.

Figure 13 illustrates the conditioned response acquisition patterns at 0.04 Mg/L and 8×10^{-3} Mg/L. Toxaphene exposure for 24 hours as compared

TABLE 3

EXPERIMENT G-1.1 GENERAL RESPONSE OF GOLDFISH TO TOXAPHENE

CONCENTRATION	TOTAL HOURS OF EXPOSURE			
	4 Hours	9.5 Hours	24 Hours	50 Hours
Control	Normal	Normal	Normal	Normal
1 Mg/L	Loss of equilibrium; disorientation*	Acute toxic response*	8 dead;remainder exhibit complete loss of equilibrium*	All dead*
0.2 Mg/L	Hyperactive	1 dead. Remainder showing signs of distress**	8 dead, 2 dying**	All dead**
4×10^{-2} Mg/L	Normal	Hyperactive	Hyperactive. 1 with complete equilibrium loss	9 dead
8×10^{-3} Mg/L	Normal	Normal	Normal?	Hyperactive
1.6×10^{-3} Mg/L	Normal	Normal	Normal	Slightly hyperactive
3.2×10^{-4} Mg/L	Normal	Normal	Normal	Normal
6.4×10^{-5} Mg/L	Normal	Normal	Normal	Normal
1.3×10^{-5} Mg/L	Normal	Normal	Normal	Normal

* Removed from toxicant to fresh water after 4 hours exposure
** Removed from toxicant to fresh water after 9.5 hours exposure

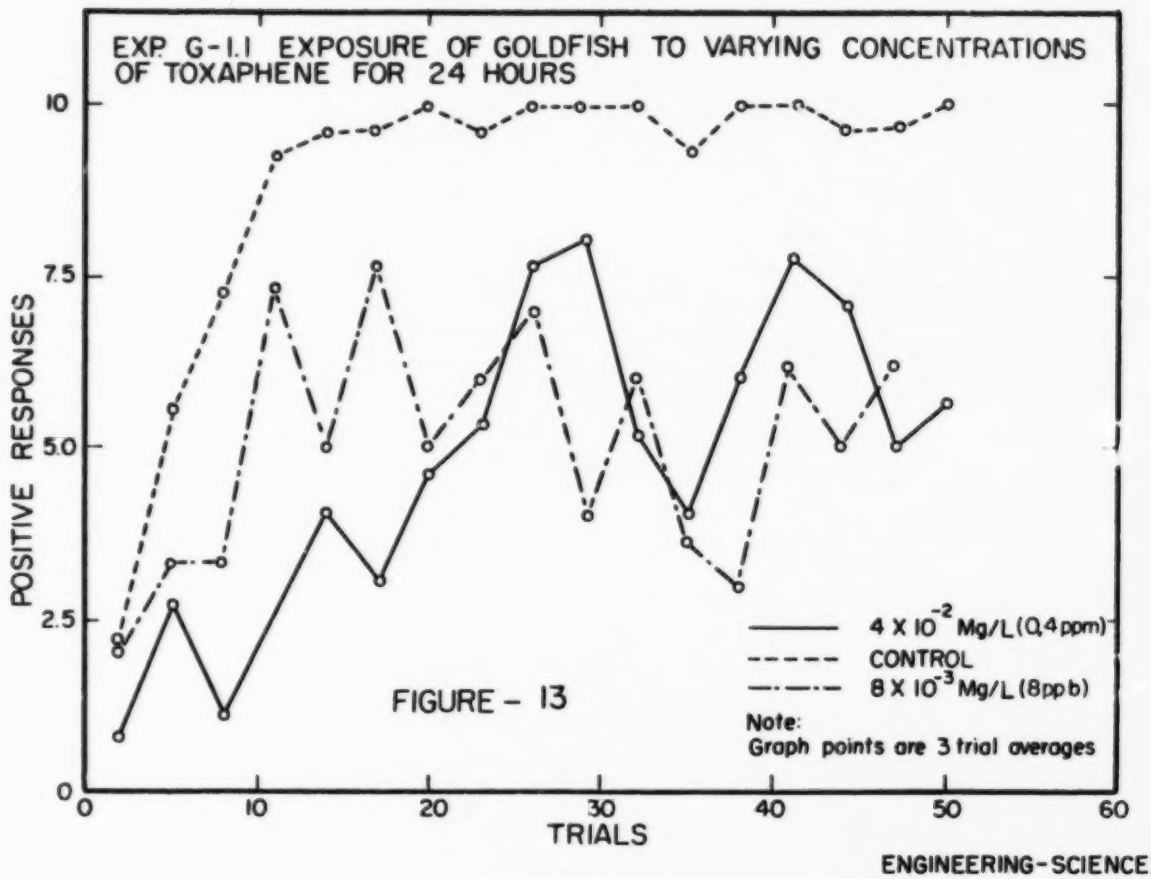

FIGURE - 13

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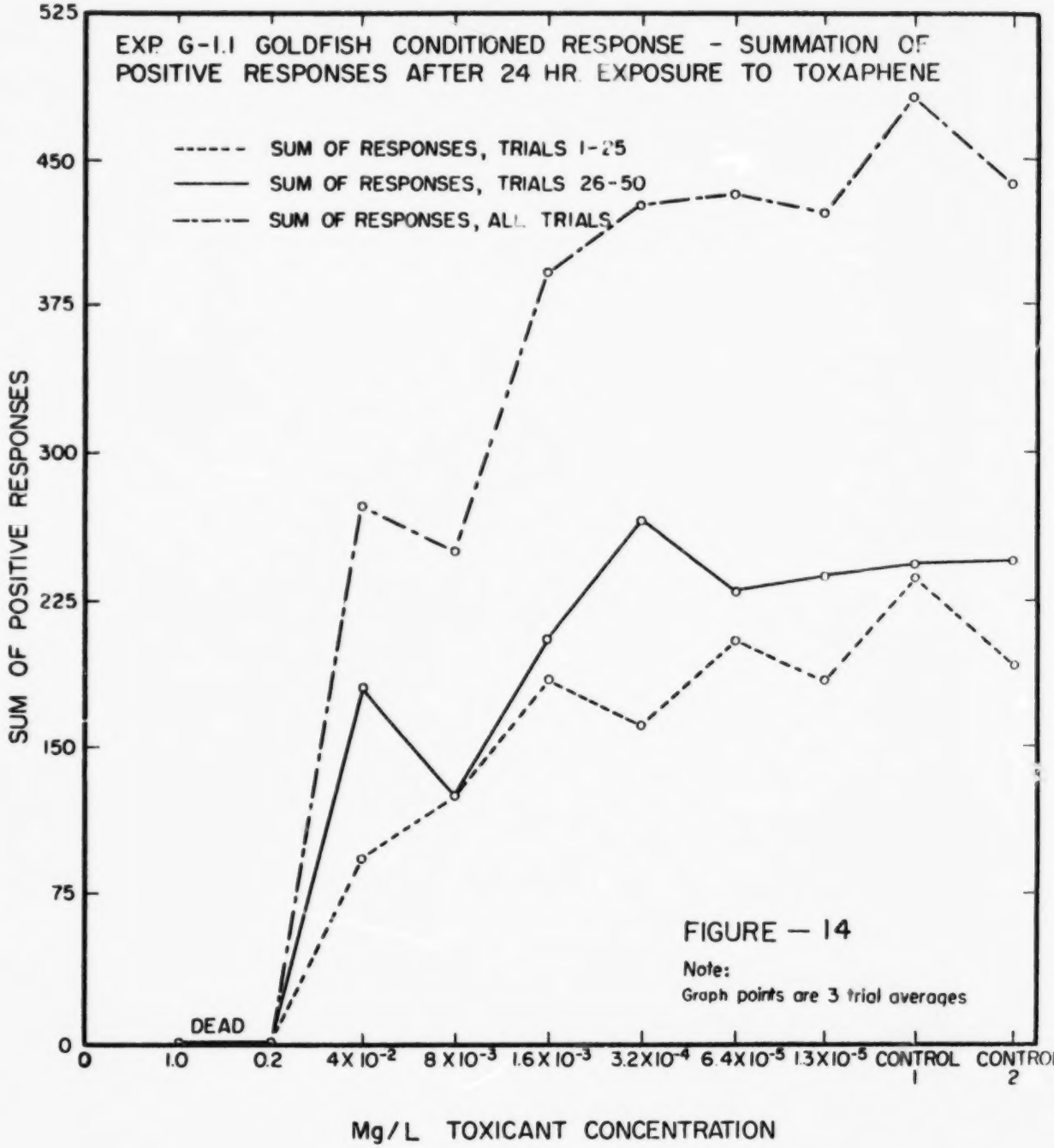
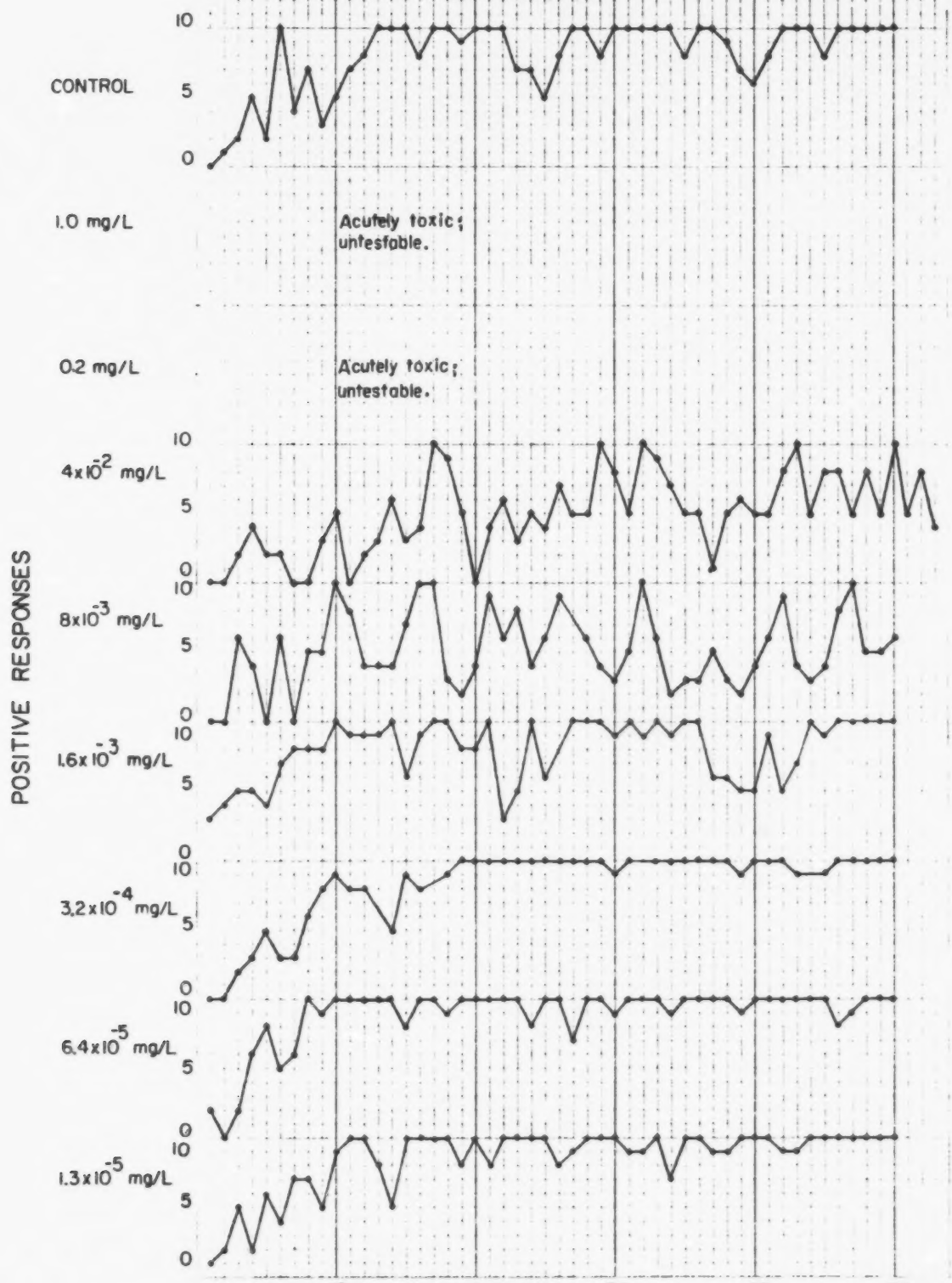


FIGURE — 14

Note:
Graph points are 3 trial averages

TABLE 4. EXPERIMENT G-II EFFECTS OF 24 HOURS EXPOSURE TO TOXAPHENE UPON THE CONDITIONED RESPONSES OF THE GOLDFISH, CARASSIUS AURATUS RAW DATA.



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to the control group. The scores for the 1.6×10^{-3} Mg/L and 3.2×10^{-4} Mg/L groups were progressively nearer that of the control, while the 6.4×10^{-5} Mg/L and the 1.3×10^{-5} Mg/L groups were not substantially different from the control.

Figure 14 presents the data in another form. As in Figure 12 of Experiment M-1 (see page 23) the total number of positive responses by fish exposed to a given toxicant concentration are plotted as 1) the first 25 trial subtotal, 2) the second 25 trial subtotal, and 3) the overall total for the experiment. The trend of increased overall performance with decreasing concentrations of toxicant is apparent.

EXPERIMENT G-1.2. CONDITIONED RESPONSE CHANGES OVER TIME IN GOLDFISH (CARASSIUS AURATUS) EXPOSED TO THE SAME CONCENTRATIONS OF TOXAPHENE

Introduction

This experiment was designed to demonstrate possible changes in responses over time in fish exposed to patently sublethal Toxaphene concentrations. One measurement also was made of a low time-concentration test group to determine the degree of response acquisition suppression under highly toxic circumstances. The other four toxicated groups were tested at 24 and 124 hours at 8×10^{-3} Mg/L toxicant, and at 25 and 125 hours at 1.6×10^{-3} Mg/L respectively.

Materials and Methods

Test Organisms

Goldfish of the "comet" strain, purchased from the Hayward Goldfish Farm, Hayward, California, were placed in the temperature-controlled laboratory aquaria and allowed to acclimatize for one week at 18± 0.5°C.

Toxicant System

The same toxicant system was used as described in Experiment M-1. Toxaphene concentrations in the toxicant tanks were as in Experiment M-1.

Toxication Procedure

Groups of fish were prepared as described in Experiments M-1 and G-1.1. The subjects were left in the toxicant for up to 5 days, then removed to the conditioned response apparatus for testing.

Testing Procedures

The testing procedure was the same as that employed in Experiment M-1, and described on page 18 of this report.

Results

Figure 15 illustrates the change in the conditioned response acquisition pattern of fish exposed for eight hours at 0.2 Mg/L (0.2 ppm). This relatively high concentration induces almost immediate symptoms of neurological disorder. It is this progressive neurological disorder which is believed to be the cause of death in acute toxicity.

At the next higher experimental concentration, 1.0 Mg/L (1 ppm), by eight hours exposure 50 per cent of the experimental group was dead, and the remainder were exhibiting symptoms of acute toxicity.

Table 5 indicates the general condition of the various test groups.

Figures 16 and 17 illustrate the changes in conditioned response acquisition patterns in toxicated groups over a five day period. These results, while preliminary, suggest the possibility that physiological adjustment or acclimatization to the toxicant may occur if the concentrations are substantially below the acutely toxic level. Since the test employed measures only the ease of acquisition of a conditioned response--which, as stated earlier, is also an indicator of the integrated functioning of the central nervous system--the possibility of continued specific toxicity at the neuron level is not ruled out. However, adjustment of the nervous system to altered response threshholds of its components (i.e. the neurons), if this is indeed the case, may indicate that chronic exposure to low levels of toxaphene is not necessarily deleterious from a neurological point of view.

A further qualification of this statement is necessary, as an exposure time of five days duration is not what is normally meant by "chronic exposure". Further tests over weeks or months of continuous low-concentration exposure to the toxicant will be necessary before any definitive statement of chronic neurological toxicity can be made.

As stated earlier, a study of the literature on toxicity of Toxaphene and other chlorinated hydrocarbons has indicated that there are probably other toxic effects than those involving increased sensitivity of the neurons. Degenerative changes in various non-nervous tissues have been reported in mammals and birds, which require other methods to elucidate than have to date been employed in this study.

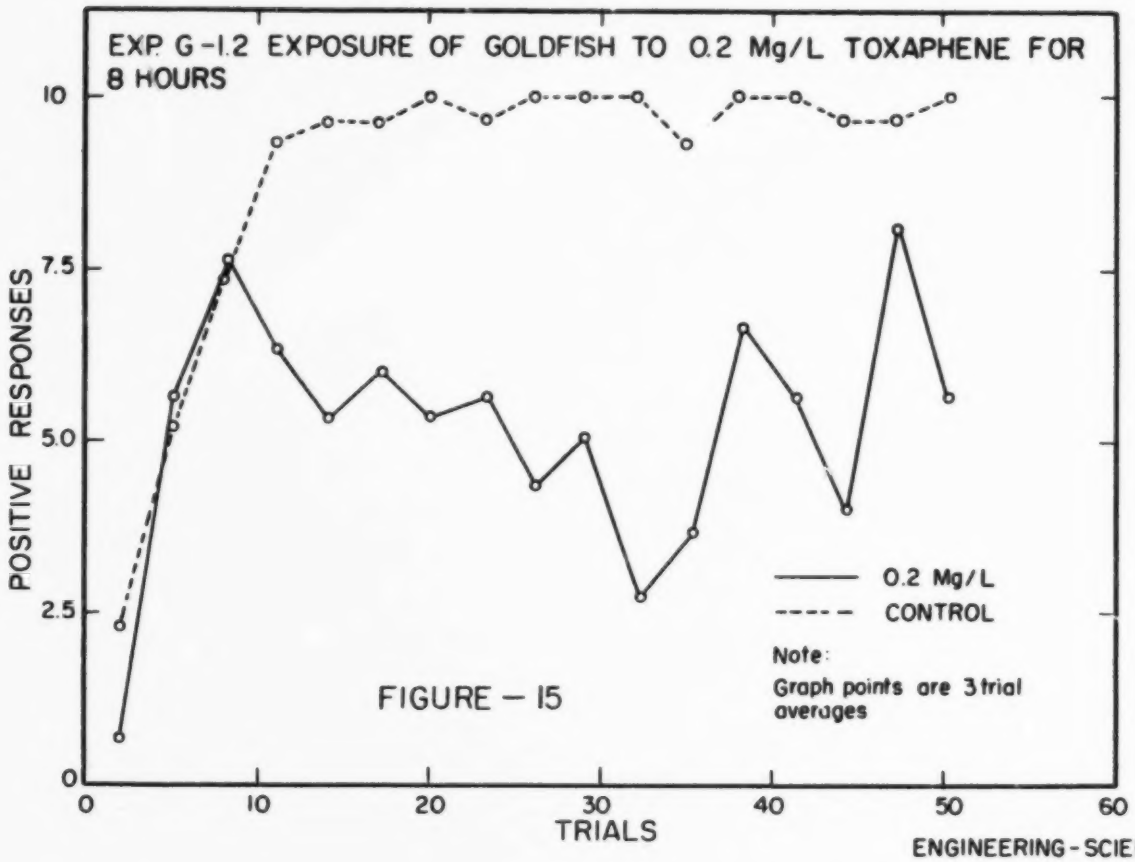
EXP. G-1.2 EXPOSURE OF GOLDFISH TO 0.2 Mg/L TOXAPHENE FOR 8 HOURS
POSITIVE RESPONSES
0
2.5
5.0
7.5
10
0
10
20
30
40
50
60
TRIALS
0.2 Mg/L
CONTROL
Note:
Graph points are 3 trial averages
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FIGURE – 15

TABLE 5

EXPERIMENT G-1.2. GENERAL RESPONSE OF GOLDFISH TO TOXAPHENE

CONCENTRATION	TOTAL HOURS OF EXPOSURE				
	3 Hours	9 Hours	24 Hours	48 Hours	170 Hours
Control 1	Normal	Normal	Normal	Normal	Normal
Control 2	Normal	Normal	Normal	Normal	Normal
1 Mg/L	Extreme toxicity	5 dead	All dead	-------	-------
0.2 Mg/L	Hyperactive; excitable	Acutely toxic response	*2 dead; apparent partial recovery in remainder		*all dead
4×10^{-2} Mg/L	Normal	Hyperactive	Hyperactive; some equilibrium loss	6 dead; equilibrium loss	all dead
8×10^{-3} Mg/L	Normal	Normal	Normal	Hyperactive	Hyperactive
1.6×10^{-3} Mg/L	Normal	Normal	Normal	Normal?	Hyperactive
3.2×10^{-4} Mg/L	Normal	Normal	Normal	Normal	Slightly hyperactive
6.4×10^{-5} Mg/L	Normal	Normal	Normal	Normal	Normal?
1.3×10^{-5} Mg/L	Normal	Normal	Normal	Normal	Normal?

* Removed from toxicant for testing at 4 hours and placed in fresh water for remainder of experiment

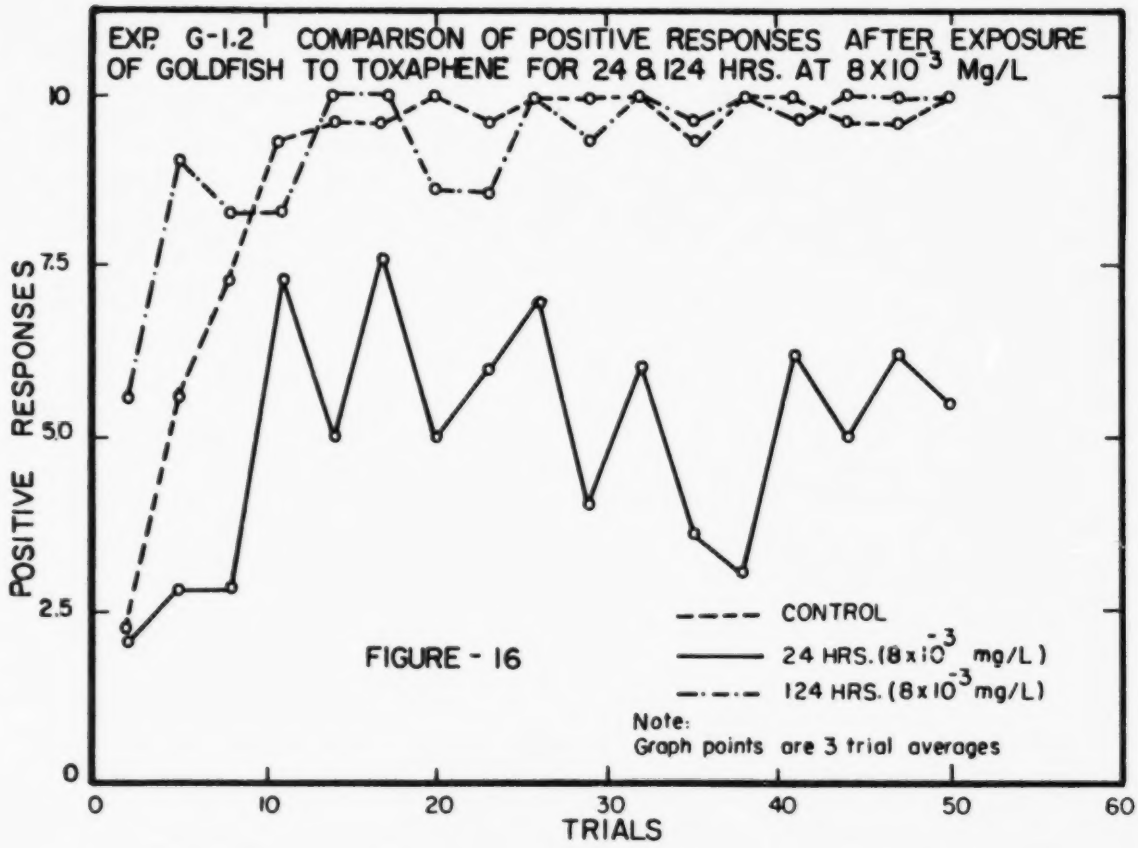


FIGURE - 16

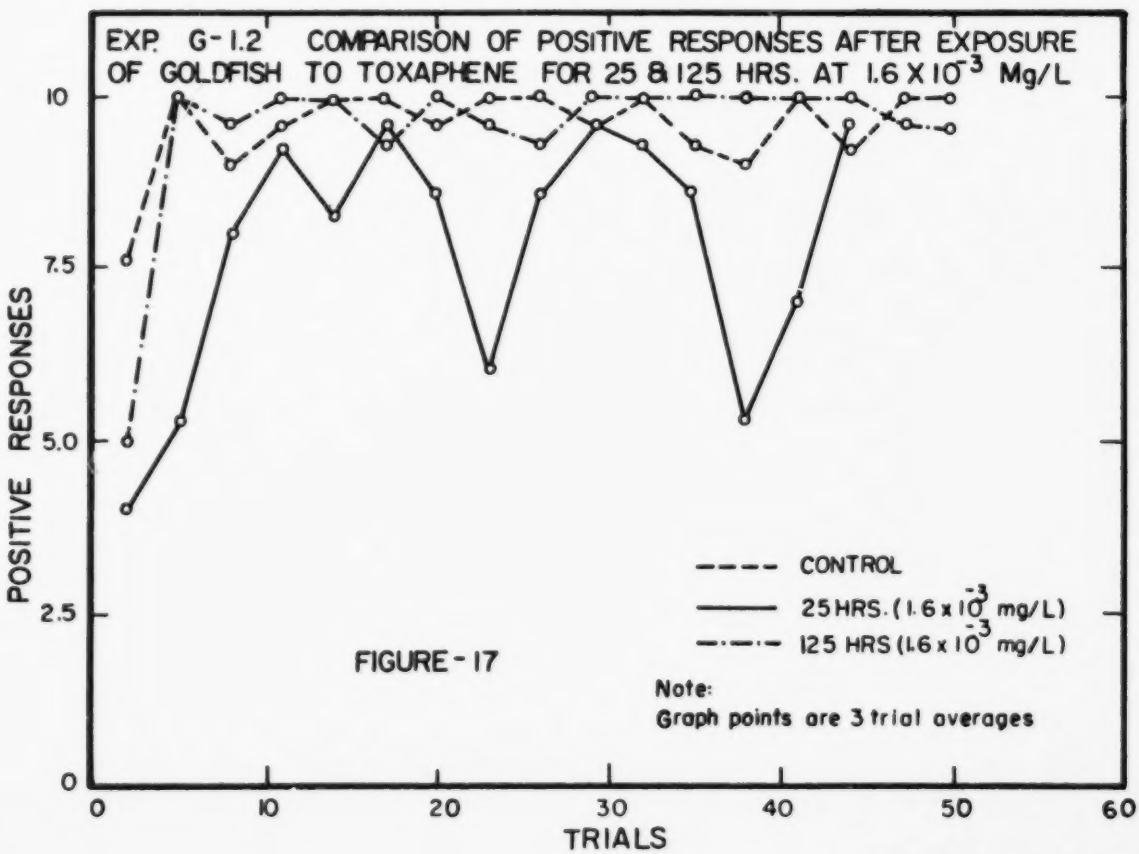


FIGURE - 17

EXPERIMENT G-2. THE EFFECTS OF 24 HOURS EXPOSURE TO PARATHION ON A SIMPLE CONDITIONED RESPONSE IN THE GOLDFISH CARASSIUS AURATUS

Introduction

The organophosphates are known to be very toxic to fishes (Rudd and Genelly, 1956). The mode of action is different from the chlorinated hydrocarbons, the toxic effect resulting from a suppression in the level of acetylcholine esterase, the enzyme which normally inactivates acetylcholine in the nervous system (Weiss, 1959). The resultant changes in nervous system function due to organophosphate toxication would therefore be expected to differ from those induced by the chlorinated hydrocarbons. This experiment was designed to explore, in a preliminary way, some of the behavioral changes due to Parathion toxication.

Materials and Methods

Test Organisms

Goldfish of the "comet" strain, procured from the Hayward Goldfish Farm, were placed in the temperature-controlled (18± 0.5°C) aquaria and allowed to acclimatize for one week.

Toxicant System

The same toxicant system was used as described in Experiment M-1. Technical Parathion of 98 per cent purity was obtained through the courtesty of the California Chemical Company, Ortho Division, Richmond, California. The concentrated material was dissolved in acetone at a ratio of 29.5 gm Parathion per liter of solvent. With the injector pipette set to deliver 2.0 ml of toxicant, a nominal T/l concentration of 5 Mg/L (5 ppm) was obtained.

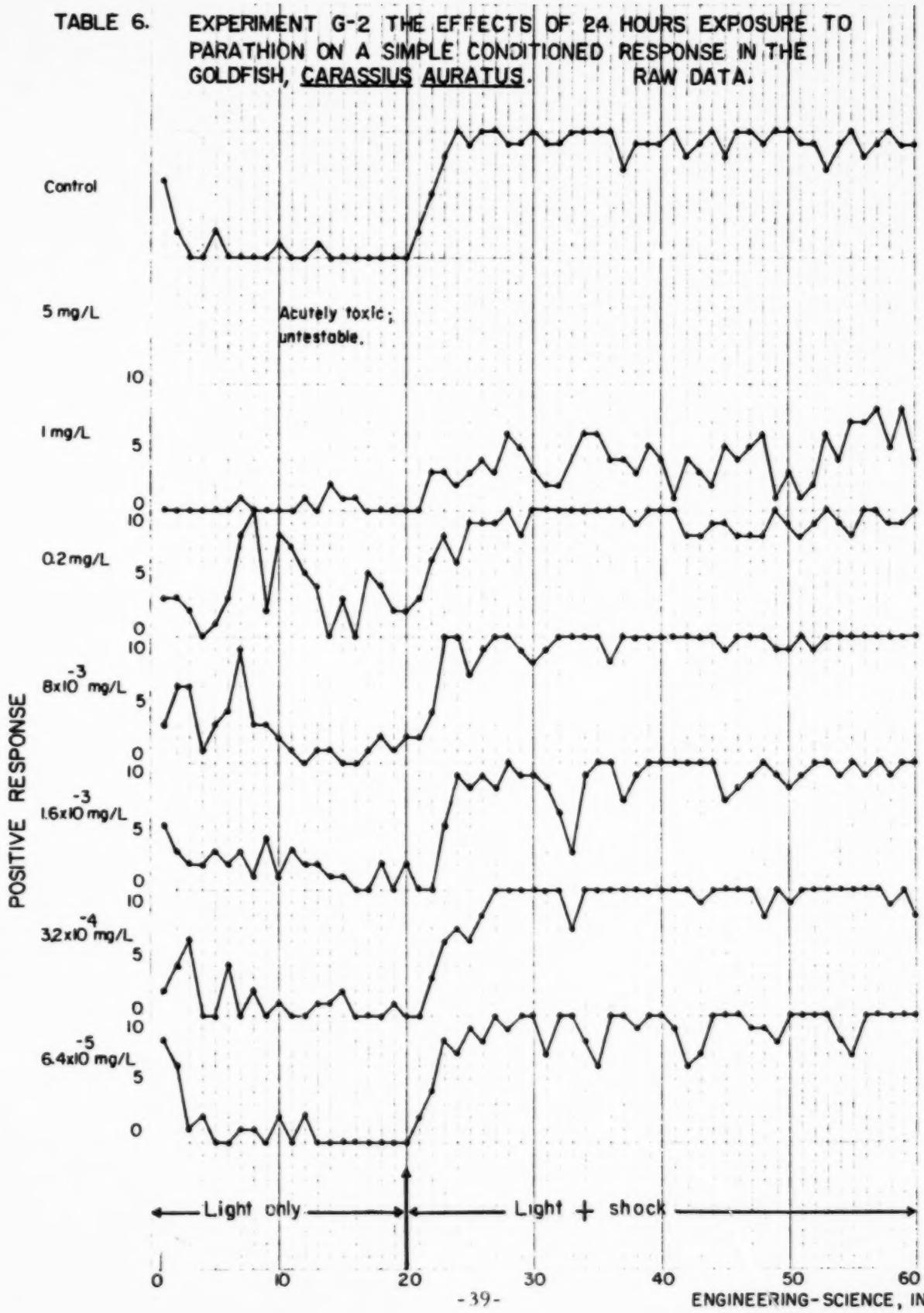
Toxication Procedures

Groups of fish were prepared as described in Experiment M-1. The subjects were then placed in the toxicant tanks for 24 hours, after which time they were removed for testing.

Testing Procedures

The testing procedure was the same as that employed in Experiment M-1, and described on page 18 of this report. An additional parameter--the response pattern with light alone--was added in this experiment, as it had become evident in previous tests that toxicated groups responded differentially to the light stimulus.

TABLE 6. EXPERIMENT G-2 THE EFFECTS OF 24 HOURS EXPOSURE TO PARATHION ON A SIMPLE CONDITIONED RESPONSE IN THE GOLDFISH, CARASSIUS AURATUS. RAW DATA.



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Results

Table 6 presents the response patterns in test and control groups. Three things are evident from the data:

1. Acquisition of the conditioned response is suppressed at higher concentrations.

2. Response to the conditioned stimulus (light) is deadened at high concentrations, as shown by the response pattern during the light only interval at the nominal 1 Mg/L toxicant level.

3. Response to the conditioned stimulus is markedly increased at lower toxicant levels, this effect diminishing as the toxicant concentrations approach zero (control).

With Toxaphene toxication the pattern is one of steadily increasing sensitivity and agitation with increasing concentration, until at very high time-concentration (T-C) functions convulsions can be readily induced.

It thus appears that there are two or more discrete response patterns to Parathion toxication:

1. Response suppression (narcosis?) at high levels and
2. Hypersensitivity to external stimuli at lower levels.

The reliability of Parathion toxic response quantification using the conditioned response parameter is not clearly demonstrated in this experiment, although the results strongly suggest the presence of concentration-dependent aberrations. Definite changes in behavior were observed. It is believed that with refined techniques satisfactory quantification of the toxic response can be achieved.

EXPERIMENT GU-1. CONDITIONED AVOIDANCE RESPONSE PATTERNS IN GUPPIES (LEBISTES RETICULATUS) EXPOSED TO PARATHION FOR 24 HOURS

Introduction

Avoidance responses form an important part of the behavioral repertoire in many animals, being especially developed in prey species. Many of these avoidance responses are innate, while others are developed in response to adverse environmental stimuli. Actually, it is likely that practically all responses have both hereditary and environmental influences contributing to their development and subsequent expression, the learned component--that is, the environmentally labile component--in general becoming more important in the

higher vertebrates.

As indicated in the earlier general discussion, fish have been demonstrated to have considerable capacities for developing environmentally directed response patterns (i. e. they learn). Thus, quantification of changes in a conditioned avoidance response due to pesticide toxication should indicate not only alterations in the general functioning and integrity of the nervous system, but suggest ways in which normal ecological interactions might be adversely affected.

Materials and Methods

Test Organisms

Guppies were obtained from the Hayward Goldfish Farm, Hayward, California, and held in the laboratory at 18± 0.5°C for two weeks before being tested. While this temperature is considerably below the optimum for guppies, no significant mortality occurred after the first week. The diverse male color patterns indicated a rather wide variety of genotypes. This is not especially desirable, but unless inbreeding for homozygous strains is undertaken there is little that can be done to reduce the genetic heterogeneity.

Toxicant System

The same toxicant system was used as described in Experiment M-1. Parathion concentrations in the toxicant tanks ranged from a nominal high concentration of 5 Mg/L to 6.4×10^{-5} Mg/L in decrements of 0.2 x C, where C is the higher concentration. The source and dilution of Parathion in Experiment G-2 (page 38 of this report) apply here as well.

Toxication Procedures

Groups of guppies were prepared as described in Experiment G-1. The subjects were then placed in the toxicant tanks for 24 hours, after which time they were removed for testing.

Testing Procedures

The conditioned avoidance response apparatus (Figure 9) was used. As stated earlier, this apparatus presents the test organisms with 1) a neutral stimulus (e.g. light) which by definition can be seen but which is innocuous); 2) an unconditioned stimulus, which in this case is the mild but distasteful electric shock; and 3) an area of sanctuary, to which the fish can flee in order to avoid the disagreeable unconditioned stimulus. To facilitate quantification and increase the complexity of the system, the apparatus was designed to alternate the locations of the various stimuli from one end of the chamber to the other. The conditioned response, the achievement of which produced a score of +1 for each fish succeeding per trial, was flight from light. That is, the cue to sanctuary from shock was the directional datum provided by the light source.

It will be recalled that the safe area was the end of the chamber farthest from the light. A score of + 1 was assigned only if the fish crossed the centerline of the apparatus while moving away from the light, and this before the onset of shock.

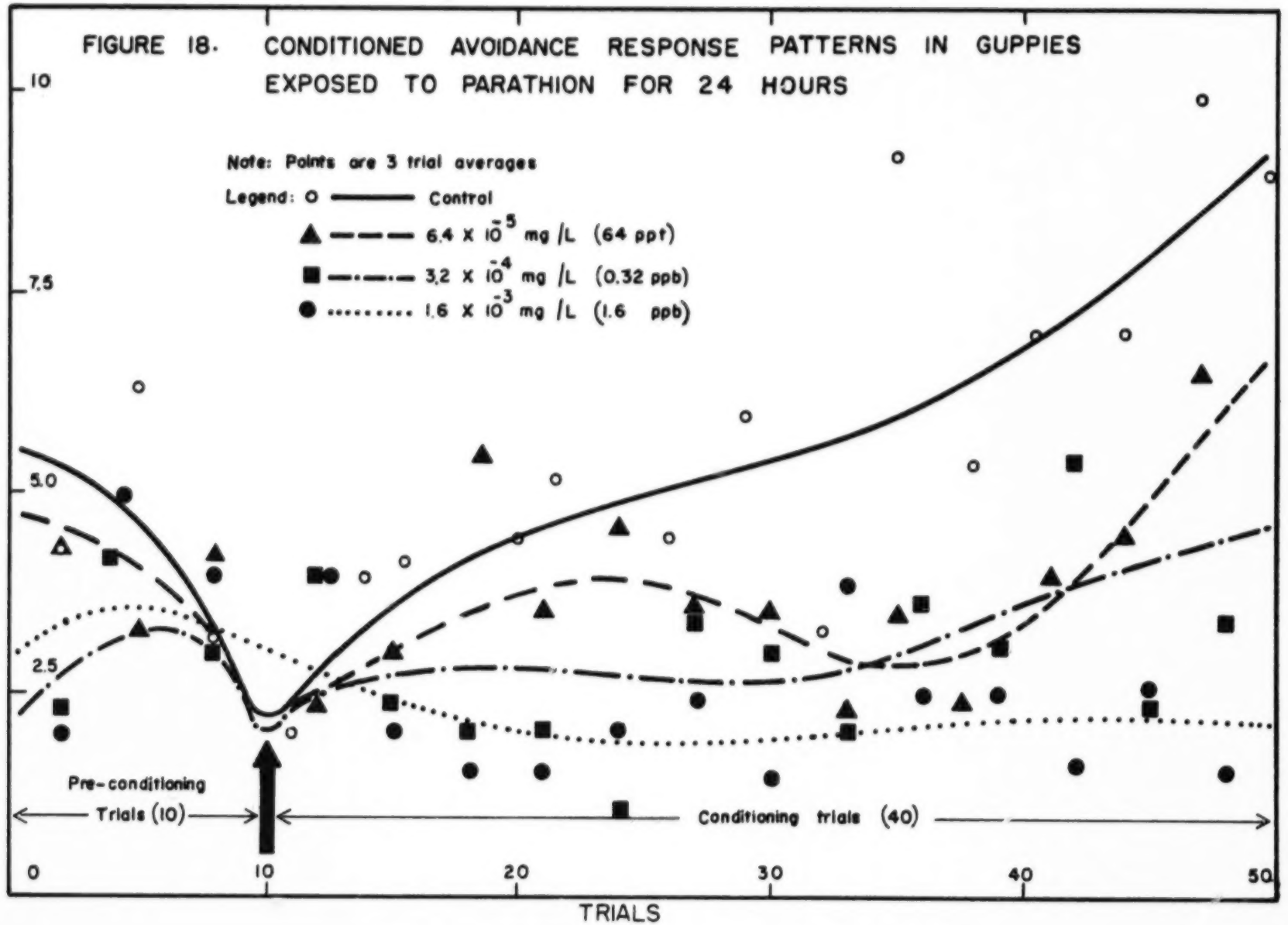
A series of pre-shock trials were run to establish the response patterns due to light alone. It should be noted that the experimental fish had already obtained previous experience in the simple conditioned response apparatus and hence were not naive to the light-shock relationship. The second portion of the experiment presented an interval of light + shock to the subjects.

Results

Significant response patterns were developed during the experiment. Figure 18 illustrates the conditioned response curves at three toxicant concentrations as compared to a control group. It will be noted that the responses of the progressively less-toxicated groups approach, but do not reach, the scores achieved by the controls.

As previously mentioned, the criterion for a positive score (crossing the apparatus mid-line) is a very conservative measure of responsiveness. Observation of the subjects while in the apparatus convincingly demonstrated that much more sensitive measurement could be made with the apparatus with suitable refinement of recording technique. However, as the purposes of the experiment were 1) to ascertain the overall utility of conditioned avoidance responses in quantifying insecticide toxication, and 2) to evaluate the merits of the apparatus, the conservative measure was retained as providing a rigorous test of response differences between test groups.

The results obtained are most encouraging for several reasons. Perhaps most important, they clearly demonstrate the quantifiability of behavioral changes due to insecticide toxication at very low time-concentration functions. In addition, the apparatus permits quantification of other very important behavioral parameters such as latency, anticipation, fatigue, etc. All of these measures will improve our understanding of the nature of toxicant action(s) on living systems. In the data presented above, avoidance responses were aggregate scores for groups of ten animals, the individuals of which were anonymous. With the improved avoidance response apparatus which is now under construction, statistical tests of within-group and between-group differences can be made with considerably smaller numbers of test organisms than is now the case.



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EXPERIMENT P-1. THE RATE AND PATTERN OF REGENERATION IN THE PLATYHELMINTH PLANARIA (DUGESIA TIGRINA) WHEN EXPOSED TO TOXAPHENE

Introduction

Regeneration rates and patterns in both invertebrates and vertebrates can be altered by various means. The process is very sensitive to metabolic poisons and can be altered by some neurotoxins as well. A more complete discussion of this subject can be found on pages 8 and 9 of this report. While the details of regenerative processes can best be followed using histologic techniques, some insight into the toxic effects of chemicals can be obtained through observation of the grosser aspects of the phenomenon. Accepting the fact that conclusions must be limited to a general statement of overall effect, it was felt that exposure of bissected planaria to organic insecticides might be instructive.

Materials and Methods

Test Organisms

Planaria (Dugesia tigrina) were obtained from the H. A. Dahl Company of Berkeley, California. The animals had been originally taken by the H. A. Dahl Company from a spring-fed, fresh water stream on the westward slope of the Berkeley Hills. The organisms were held in aerated fresh water without temperature control for four days prior to use, and were fed once on fresh liver in the interim. Individuals used in the experiment averaged 1 cm in length when normally extended.

Toxicant System

The toxicant system used was that described in Experiment M-1. A holding chamber was designed which permitted groups of test animals to be floated in the toxicant tanks and examined at will. Figure 19 illustrates the major elements of this simple unit. The use of clear plexiglas tubing in the body permits detailed study of the organisms under the binocular dissecting microscope while still bathed in toxicant. To accomplish this a cork is inserted into the open end of the tube. The cork, together with the fine mesh nylon cloth at the lower end of the tube, prohibits entry of air and permits retention of the contained toxicant while the organisms are examined. The nylon cloth (parachute fabric in this case) is fine enough to prevent the planaria slipping through the meshes. The animals are unable to escape out the open top of the tube because they require a wet surface on which to travel, and the exposed portion of the tube remains dry.

Toxicant exchange was assured by flushing the tubes at least once daily; this being accomplished by simply raising the tube above the toxicant tank, allowing the contained liquid to drain out, and replacing the tube in the tank

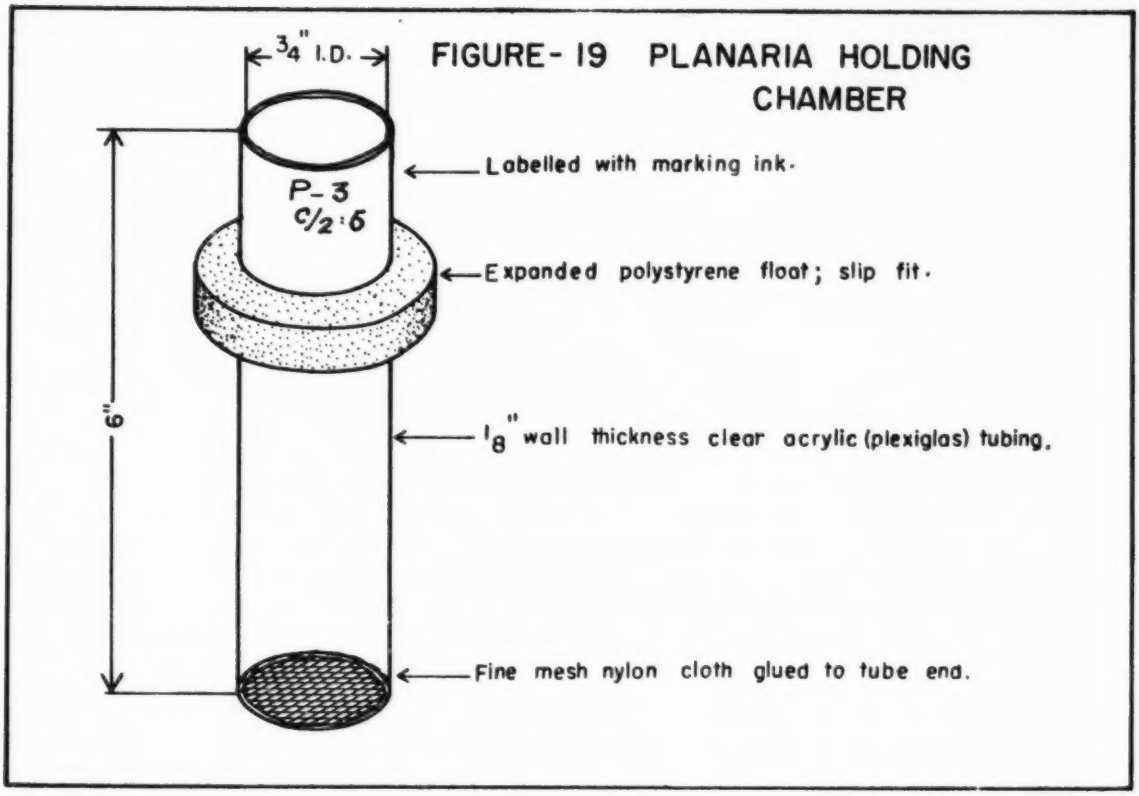

FIGURE- 19 PLANARIA HOLDING CHAMBER

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where it refilled of its own accord, gradually sinking until detained by the polystyrene float.

One possible disadvantage of this unit is that while the toxicant inside and outside the tube is in contact through the nylon cloth, there is no forced replacement of the liquid contained in the chamber. This feature, with the attendant possibility of local absorption or adsorption conditions within the tubes, might tend to reduce the otherwise advantageous features of the continuous flow apparatus. Improvements to preclude this possibility are planned.

Toxication Procedures

Twenty groups of test animals were prepared by the following method:

1. A planarian was removed from the stock culture and placed on a polyethylene pad.

2. The animal was bissected with a sharp scalpel while extended.

3. Head and tail halves were placed in separate holding chambers.

4. This procedure was followed until 10 tubes of head ends and ten of tail ends had been obtained, the bissected animals being randomly distributed among the chambers to assure uniformity of test groups.

5. The planaria holding chambers were then placed in the experimental toxicant tanks, where they floated vertically with the mesh-enclosed end down, the depth of submergence being determined by placement of the polystyrene float. The planaria moved freely about in the toxicant solution contained within the tubes.

Testing Procedures

The planaria holding chambers were periodically stoppered with a rubber cork and removed from the toxicant tanks for observation under the dissecting microscope. The general movement and behavior patterns were studied in detail to determine any useful quantifiable aspects, and the state of regeneration of the organisms evaluated. Observations on regeneration state were limited to the gross and macroscopic level; for instance, whether new tissue was forming, whether eye spots had developed, etc. The use of histological comparison has much to recommend it here, as many of the important toxicant-induced changes would be most readily discerned at the tissue level. In this experiment, however, only grossly observable changes were recorded. The periodic observations were continued until regeneration was completed.

Results

Table 7 presents the results of this preliminary experiment. It will be noted that at 1 Mg/L (1 ppm) and 0.2 Mg/L (0.2 ppm) Toxaphene concentration, regeneration was entirely suppressed, the animals dying at the higher concentration.

The remaining groups were progressively less suppressed, with 1.3×10^{-5} Mg/L (13 ppt) only slightly (perhaps insignificantly) impaired.

TABLE 7

REGENERATION RATE OF PLANARIA (DUGESIA TIGRINA) EXPOSED TO VARIOUS CONCENTRATIONS OF TOXAPHENE AT 18°C

Group	Mg/L Toxaphene	Days to Regeneration	Group	Mg/L Toxaphene	Days to Regeneration
T/1	1.0	*--	T/6	3.2×10^{-4}	14
T/2	0.2	**--	T/7	6.4×10^{-5}	13
T/3	4×10^{-2}	+16	T/8	1.3×10^{-5}	10
T/4	8×10^{-3}	+16	Control	--	9
T/5	1.6×10^{-3}	16	Control	--	9

* Animals disintegrating by Day 8. By Day 11, all animals dead and disintegrated.

**Negligible regeneration. Cut ends closed over and rounded. No growth, but no mortality.

\+ Aberrant.

This bioassay technique appears to have considerable merit, and should be studied more carefully. The one notable disadvantage is the length of time required for completion of the test. This can be reduced 50 per cent by increasing the test temperature. The unique data which it provides may render it extremely valuable for baseline studies of compounds whose specific toxic properties are unknown.

The "time to completed regeneration" is only one of the many parameters of this complex phenomenon. Other measures--rate and pattern of cell growth, activity level and pattern, respiration rate, etc.--may prove as or more sensitive than overall regeneration time.

EXPERIMENT H-1. THE RESPONSE OF THE COELENTERATE HYDRA (PELMATOHYDRA OLIGACTIS) TO GRADED CONCENTRATIONS OF TOXAPHENE

Introduction

This experiment was designed to evaluate the general nature of the toxic response of a very primitive kind of animal. Since aquatic ecosystems have a larger representation of primitive invertebrates than terrestrial ecosystems, and since these simpler forms often contribute materially to the dynamics of aquatic ecosystems, insight into their sensitivity to toxicants seemed pertinent.

Materials and Methods

Test Organisms

Hydra (Pelmatohydra oligactis) were obtained from the H. A. Dahl Company, Berkeley, California. The Dahl stock had been initially obtained from an unknown location in the San Joaquin Valley, California, and maintained as a reproducing colony from which our individuals were taken. The hydra were held in the laboratory for several days in unheated aquaria whose mean temperatures were 19°C.

Toxicant System

The same toxicant system was used as described in Experiment M-1. Toxaphene concentrations in the experimental aquaria were as in Experiment M-1.

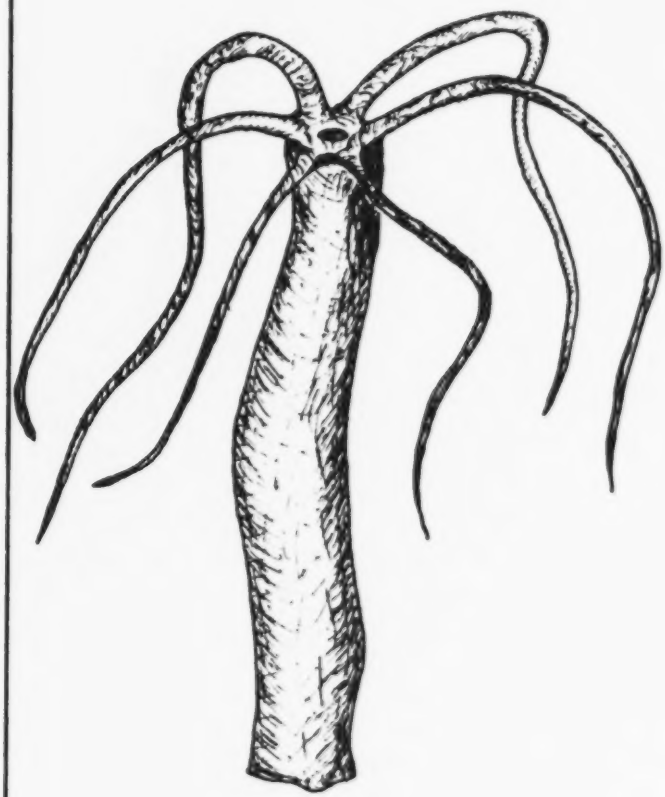
Toxication Procedure

Fifty milliliter Pyrex beakers were labelled and partially filled with toxicant from the continuous flow toxicant tanks. Sufficient liquid was added to the beakers to cause them to float in the toxicant tanks, with sufficient "freeboard" to prevent sinking if jostled while floating. A total of 10 groups of 10 individuals each (eight experimental groups, 2 control) were prepared. The contained toxicant was changed by decantation once each day, generally after each observation period.

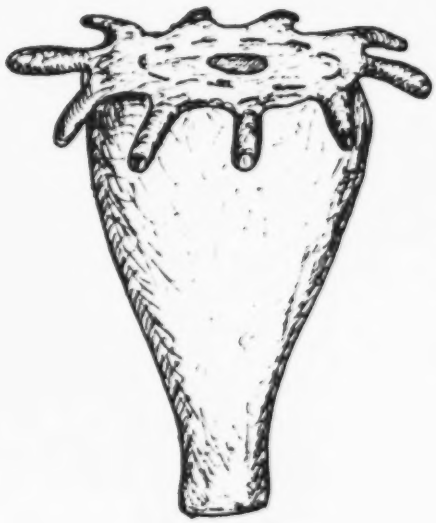
Testing Procedures

Daily observations were made on general condition, feeding ability, and sensitivity to various stimuli (light, gentle touching with a glass rod, vibration, heat). Observations were made first while the organisms were undisturbed and still in the toxicant tanks, and then after the beakers had been removed to the dissecting microscope.

FIGURE 20. NORMAL AND TOXAPHENE-TOXICATED POSTURES OF HYDRA (PELMATOHYDRA OLIGACTIS)



NORMAL



ACUTELY TOXICATED
0.2 Mg/L (0.2 ppm) for 24 hours

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Results

Definite response patterns were noted, with increasing aberrancy at the higher concentrations of toxicant. At above 8×10^{-3} Mg/L (8 ppb) Toxaphene concentration, all feeding activity was suppressed. Responsiveness to external stimuli was dampened, then inhibited at the higher concentrations. Posture, general appearance, and orientation in the beakers also were altered. A characteristic posture was assumed by individuals exposed to the higher concentrations. Figure 20 indicates the normal and acutely toxicated postures of hydra.

At concentrations below those producing an acute toxic response, the organisms exhibited partial loss of control over tentacular movement, contractility, and were less sensitive to external stimuli.

However, precise quantification of these changes was not attempted. In general the organisms seemed less acutely sensitive to Toxaphene than the fish species tested and their responses more difficult to objectively quantify.

The experiment was not continued over a sufficiently long period to determine whether or not Toxaphene has chronic or cumulative effects on important life processes of hydra. It would be of value to ascertain whether sexual or asexual reproduction (by budding in this form) is impaired. The results of Experiment P-1, wherein regeneration in planaria was impaired, suggest that budding--a process somewhat akin to regeneration--might be adversely affected.

In summary, this and other similar tests made on hydra indicate that its sensitivity to acutely toxic levels of Toxaphene is substantially lower than fish, Daphnia, and other higher forms. No statement on chronic toxicity can be made. Studies to determine this would be desirable.

EXPERIMENT G-3. TOTAL ACTIVITY CHANGES IN THE GOLDFISH (CARASSIUS AURATUS) DUE TO TOXAPHENE TOXICATION

Introduction

This exploratory experiment was designed to 1) test the total activity apparatus described on page 19 of this report, and 2) demonstrate whether the quantification of total activity would be useful in assessing the toxication of fishes by Toxaphene. The results are considered preliminary, and useful primarily in improving the sensitivity of the procedure.

Materials and Methods

Test Organisms

Goldfish of the "comet" strain from the Hayward Goldfish Farm were

acclimatized for one week in the laboratory at 18± 0.5°C.

Toxicant System

The toxicant system used was that described in Experiment M-1. Toxaphene concentrations were as in Experiment M-1.

Toxication Procedures

Randomly selected groups of 10 goldfish were prepared as previously described and placed in the toxicant tanks.

Testing Procedures

After varying intervals of exposure to the toxicant the mesh baskets of fish were removed to the total activity apparatus which contained control (fresh) water at 18.0°C. After a 30 minute stabilization interval, the counting device, which consists of light source, photo-electric sensing unit, relay, and electro-magnetic counter (see Figure 10) was activated. The apparatus recorded one count when the light beam was interrupted and then restored. Consequently, scores accumulated for the test groups were indicative of the total amount of bodily (individual whole animal) movement within the chamber. The device was not sensitive enough to record movements of individual fins or opercula.

Results

Because of adjustments to the apparatus between some of the runs, absolute values for some of the toxicant groups were not obtained. However, a sample of the useable data, compiled in Table 8, indicates the range of total activity resulting from Toxaphene toxication. These scores were all recorded at one setting of the apparatus and are hence comparable.

TABLE 8

30 MINUTE TOTAL ACTIVITY SCORES AT 18°C OF GOLDFISH TOXICATED WITH TOXAPHENE

TOXICANT CONCENTRATION	TOTAL HOURS TOXIC EXPOSURE	TOTAL ACTIVITY SCORE
Control	0.0	62
1 Mg/L	0.5	462
1 Mg/L	1.0	1,064
0.2 Mg/L	5.0	632
1.6×10^{-3} Mg/L	5.0	93

The trend toward increased total activity with increased Time-Concentration functions is apparent. There is little doubt that the increases-occurring at

a given concentration but with greater duration of exposure, and with exposures of equal duration but with increasing concentrations (both producing higher T-C functions)--reflect a state of progressive toxicity.

Refinement of the apparatus is necessary to permit testing of smaller organisms (e.g. guppies). Increased sensitivity would also be desirable although, as the above figures indicate, score differences great enough to be determined without the use of statistical tests are already possible. It is believed that this parameter of the toxic response merits further development.

V. CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Toxicity Parameters and Ecological Linkage

As is evident from the report, the bulk of the year's activity has been devoted to 1) a search for biologically meaningful parameters of the organic pesticide toxic response; and 2) development of apparatus and techniques capable of objectively quantifying those parameters which seemed promising. Of the great variety of toxic response components which are known or can be inferred from the literature, the conditioned response, regeneration pattern, and total activity appeared most worthy of development. Prominent among the reasons for this conclusion is the persistent demand by those working in applied aspects of pollution biology and control that toxicity measures be linked to ecological effects. And while this is an understandable request, it is extraordinarily difficult to fulfill. For there are thousands of discrete biological mechanisms functioning interdependently within any living system, all necessary in varying degrees for the maintenance and continuance of the individual and the population. Persistent interruption of one or more of these mechanisms may--depending upon the importance of the mechanism, the degree of impairment, the presence or absence of compensatory pathways, homeostatic resources, etc. --induce maladaptive alterations in the overall performance of the organism to an extent that changes occur in the ecosystem. The selection of ecologically significant parameters therefore has its complications.

The Conditioned Response

The conditioned response is a measure of performance. The quantification of the time (or trials) required to develop a conditioned response is a rate measurement. Other workers have attempted to measure other rates; for example filtration rate, pleopodal beating rate, opercular movement rate, etc. The seeming difference in these functions as compared with the conditioned response is that the former are more or less continuous processes, while the latter is an intermittent one. This is equally true of regeneration, which is an intermittently utilized physiological mechanism. On the other hand, component

parts of the complex mechanisms which we have labelled "conditioned response" and "regeneration" are constantly utilized by living systems. The critical point is not whether the function being measured is continuous or intermittent. Rather, the paramount question must be: Does the change in rate reflect a significant maladaptation of the organism as a result of toxication? Or to the contrary, is it 1) a function the loss or reduction of which, because of compensatory mechanisms, becomes unimportant to the organism, or 2) a function which, though readily quantifiable, is insensitive to the effects of the specific toxicant in question?

Any worthwhile bioassay must satisfy these and other requirements. It is concluded that the conditioned response does so. The preliminary studies reported herein have demonstrated several signficant points:

1. Conditioned avoidance response acquisition patterns can be quantified in fish. Change in this acquisition rate can be a useful tool in assessing the normality of living organisms.

2. Toxication due to organic pesticide poisoning significantly alters the conditioned response acquisition patterns, the degree of alteration being directly related to the Time-Concentration (T-C) function.

3. Simple conditioned responses, while readily quantifiable, seem less useful than the more complex conditioned avoidance responses. It is possible that, once sufficient understanding has been gained in the role of toxicants on conditioned response acquisition and behavior generally, the development of convenient tests using simple conditioned responses will become practical. The use of avoidance, or the directed aversive behavior elicited in the escape reaction, will permit subjection of the bioassay to ecological evaluation, should this prove necessary or desirable.

4. With the development of appropriate apparatus (now in preparation) aversive behavior can be measured with considerable accuracy, and certain important components of the general response can also be determined. Latency (the period between onset of stimulus and beginning of response), anticipation (onset of response prior to receipt of the stimulus), and extinction (the decay of an integrated response due to removal of the unconditioned stimulus) are exceptionally good indicators of nervous system condition. Multiple measures are especially valuable because the specific toxic effects of even such chemically similar compounds as DDT, Endrin, and BHC are not identical. With a sufficient repertoire of sensitive behavior measures, these differences can be distinguished.

5. The toxic response patterns of chlorinated hydrocarbons and organophosphates, despite both classes of toxicants being neurotoxins, are distinctly different. Detailed observations on the subjects throughout the reported series of experiments indicated that goldfish exposed to

Toxaphene became progressively more hyperactive with increased T-C functions. At very high T-C functions, the mild shock produced convulsions. Conversely, Parathion toxication induced a "lethargy" at higher T-C functions, although the fish responded in a hypersensitive manner to external stimuli (see pre-shock interval of Table 6). At very high T-C functions a state of "narcosis" prevailed, with all responses depressed.

Regeneration

Regeneration, as a process, may be quantified in terms of rate of regrowth. It may also, with proper microscopic technique, be used to determine the presence or absence of specific biological mechanisms. That is, the various activities composing the process of regeneration can be examined to ascertain their presence, absence, or relative aberration. The studies conducted to date indicate the following specifics relative to the process.

1. Regeneration in platyhelminths is adaptable to bioassay studies.

2. The organic pesticides exhibit toxic effects upon the process. Toxaphene has a markedly greater adverse effect than does Parathion, the specific reasons being obscure at present.

3. Quantification of the gross features (i.e. time to complete regeneration) by macroscopic observation is not a sensitive enough measure of toxicity. Histological studies of regenerating organisms will yield further information on specific toxic effects, and provide a basis for more accurate determination of rate of regrowth. Detailed studies of the process may ultimately permit the use of the grosser technique as a bioassay.

Total Activity

Preliminary total activity measurements suggest that this parameter may be a very useful tool in quantifying toxic responses. Increases of up to sixteen fold in total activity have been recorded with Toxaphene toxication. With further refinement of technique greater sensitivity is expected. The greatest utility of this measure appears to lie in correlating it with other response parameters. By thus developing a toxic response syndrome--which is a direct manifestation of the toxic response sequence discussed earlier--the effects of compounds whose toxic natures are undefined (e.g. complex pulp mill wastes) can be explored in depth.

RECOMMENDATIONS

On the basis of the above the following recommendations are made:

1. That work be continued on the conditioned avoidance response as a

promising bioassay of water pollutants;

2. That other parameters such as organism weight, respiration rate, reproductivity, etc. be developed simultaneously to provide a more comprehensive picture of the general status of the organisms exhibiting aberrant conditioned responses.

3. That a standard laboratory animal be developed for bioassay use. It is recommended that guppies(Lebistes reticulatus) be inbred to reduce the degree of genetic heterogeneity now obvious in commercially available stock, and that further work utilize these as baseline organisms. Comparative statements of the toxicity of water pollutants to other species can then be made.

4. That the histologic aspects of regeneration be explored in greater detail. This may not be possible within the current contract framework because of the degree of specialization involved.

5. That further refinements in the total activity apparatus be made, and where possible the measure be correlated with other toxic response parameters. The integration of such data will ultimately permit us to define pollution effects in terms of toxic response syndromes.

6. That additional toxic response parameters be explored concomitantly with the above research efforts. Behavioral measures requiring shorter test periods are especially needed.

7. That an appreciable part of the research effort be devoted to determining the dynamics of toxic responses. This can be in part accomplished by appropriate design of the planned experiments. Where possible, effort should be directed toward examining the theories of toxic response threshold , the T-C (Time-Concentration) function, and other basic toxicology problems. For only through an understanding of these basic and as yet ill-defined concepts will we be able to develop bioassays with useful predictive power and biological significance.

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